QUALITATIVE AND QUANTITATIVE ANALYSIS OF ACONITINE ALKALOIDS IN CHINESE MEDICINAL MATERIALS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRY

by

Kwok Chiu Nga
(郭超芽)

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY

THE CHINESE UNIVERSITY OF HONG KONG
Department of Chemistry

August 1998

The members of the Committee appointed to examine the dissertation of C. N. KWOK:

Paul P. H. But (Chair)

Dominic T. W. Chan

Jimmy C. M. Yu

H. W. Leung

TABLE OF CONTENTS

ABSTRACT

Aconite is an important class of traditional Chinese medicine (TCM) and is prepared from the roots of certain species of Aconitum. For instance, "Chuanwu" and "Caowu" are the processed aconites from the root of the plants Aconitum carmichaeli and Aconitum kusnezoffii, respectively. Since the raw root of Aconitum plant contains several highly toxic aconitine-type alkaloids, such as aconitine, hypaconitine, and mesaconitine, they are usually processed by heating, steaming and soaking in order to hydrolyze these aconitine analogues to the much less toxic benzoylaconine analogues. However, these processing procedures are not necessarily standardized; and the alkaloid composition, and consequently the toxicity or the therapeutic effectiveness, of the aconite varies substantially. It is, therefore, essential to develop a precise and highly sensitive analytical method to monitor the residual amount of the aconitine-type alkaloids in the processed Aconitum roots.

In the present study, a highly selective and sensitive analytical method based on on-line high performance liquid chromatography and atmospheric pressure ionization mass spectrometry (HPLC/APIMS) has been developed to identify and quantify aconitine, hypaconitine, and mesaconitine contents in commercially available Chuanwu and Caowu. The alkaloids are first separated and purified from the complex matrices by multiple extractions with diethyl ether. The pre-concentrated alkaloid extract is then separated using reversed-phase HPLC from each other and from other residue matrices. The separated alkaloids are sequentially analyzed by a UV detector and by an on-line mass spectrometer. Atmospheric pressure ionization techniques are used to couple the HPLC outlet to the source region of the mass spectrometer.

For the liquid chromatographic separation, a mobile phase consisting of 77 % buffer, 13 % acetonitrile, and 10 % tetrahydrofuran, whereas the buffer contains 0.5 % triethylamine, 50 mM ammonium acetate, and 1 % acetic acid, is found to provide optimal retention and separation. Good separation between aconitine-type alkaloids and the residue matrices were obtained within 30 minutes of chromatographic run.

Both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) methods have successfully been used to couple the HPLC outlet with a triple-stage quadrupole (TSQ) mass spectrometer. Intense protonated molecule-ion signals $[M+H]^+$ were generated under either APCI or ESI conditions. With the use of dimethylaminobenzaldehyde (DAB) as internal standard, the aforementioned alkaloids could be identified and quantified over a range of 300 picograms to 7.5 micrograms. The detection limit of the alkaloids using this method was roughly 75 picograms.

摘要

烏頭是一類常用的傳統中藥,例如製川烏及製草烏,是烏頭經過加熱、加蒸氣、浸泡等工序而製成。炮製過程降低了其中主要有毒成份－烏頭鹼（aconitine）、中烏頭鹼（mesaconitine）、次烏頭鹼（hypaconitine）等類生物鹼。由於炮製工序沒有劃一標準,導致藥物中所含生物鹼的組成及含量變化很大。爲了確保藥物的療效及安全性,必須發展一種精密、準確的分析方法,用於測定烏頭樣品中的生物鹼含量。

本文使用高效液相色譜大氣壓下離子化質譜法作爲一種高精密度、準確度的分析方法,分析川烏及草烏中烏頭鹼、中烏頭鹼、次烏頭鹼的含量。首先用乙醚提取烏頭樣品中的生物鹼,然後用高效液相色譜儀分離個別的生物鹼,最後用紫外光檢測器及線上質譜儀同時測定這些生物鹼的含量,當中需使用大氣壓下離子化技術才能連接高效液相色譜儀及質譜儀。

流動相中以水溶液－乙腈－四氫呋喃（77：13：10）的分離效果較好,當水溶液中含有0.5%三乙胺,50mM乙酸銨及1%乙酸,烏頭中的生物鹼能夠在三十分鐘內被完全分離,另外大氣壓化學游離法（APCI）及電灑法（ESI）都能用於連接高效液相色譜儀及三段式四極質譜儀（triple-stage quadrupole）,生物鹼在APCI及ESI能產生穩定的離子〔M+H〕$^+$,加上內標二甲基苯甲醛後生物鹼的線性檢測範圍爲300pg到7.5μg,本方法的最低檢測極限爲75pg。

LIST OF FIGURES

LIST OF TABLES

ABBREVIATION

A	Aconitine
ACN	Acetonitrile
amu	atomic mass unit
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
CI	Chemical ionization
CID	Collision-induced dissociation
DAB	Dimethylaminobenzaldehyde
DAD	Diode-array detector
EI	Electron ionization
ESI	Electrospray ionization
FAB	Fast atom bombardment
GC/MS	Gas chromatography coupled with mass spectrometry
HA	Hypaconitine
HPLC	High performance liquid chromatography
HPLC/APIMS	High performance liquid chromatography coupled with atmospheric pressure ionization mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MA	Mesaconitine
MS	Mass spectrometry

MS/MS	tandem mass spectrometry
MSMSC	Correction factor of tandem mass spectrometry
m/z	mass-to-charge ratio
PB	Particle beam
PSO	Psoralen
PW	Peak width
Q1	Quadrupole near ion source
Q3	Quadrupole near ion detection system
Q2	Octapole between Q1 and Q3
R	Resolution power between two adjacent peaks
RF	Radio frequency
RSD	Relative standard deviation
SIM	Selected ion monitoring
TCM	Traditional Chinese medicine
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TSP	Thermospray
TSQ	Triple-stage quadrupole
UV	Ultraviolet light

CHAPTER ONE

RESEARCH BACKGROUND

1.1 Introduction

1.1.1 Alkaloids

The term "alkaloids" applies to a very large group of compounds that occur almost exclusively in plants, and are widely distributed throughout the plant kingdom. These compounds contain at least one nitrogen atom which, in turn, constitutes part of the heterocyclic ring in a majority of the alkaloids. Because of the presence of this heterocyclic nitrogen, most of the alkaloids are basic in solution. The pKa values vary from about 6-12, with most alkaloids in the range of 7-9. In general, the free base is soluble in organic solvents and not in water. Therefore, the method for the isolation of alkaloids are based upon the fact that they can be extracted under neutral or basic conditions as free base with organic solvents [1].

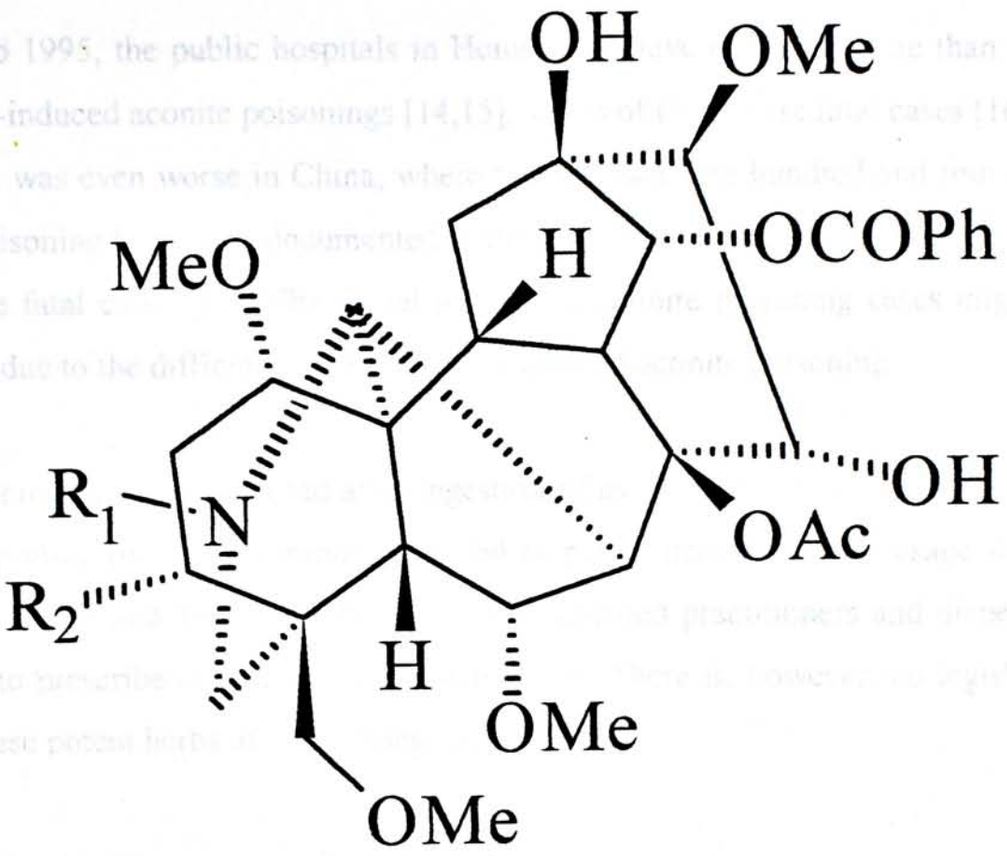
Similar to most of the natural products, there is no systematic nomenclature for alkaloids. Each compound is referred to only by a trivial name. Almost all of the trivial names for alkaloids end in an "-ine". Since there is no rigid system of classification of alkaloids on a structural basis, the alkaloids are classified by the heterocyclic system from which a group of compounds may formally be derived and/or by the plant species from which a class of alkaloids is isolated. Examples of the main classes of alkaloids are: pyrrolidine, piperidine and pyridine alkaloids; isoquinoline and tetrahydroisoquinoline alkaloids; quinoline alkaloids; indole alkaloids; diterpenoid and steroid alkaloids [2].

1.1.2 Diterpenoid alkaloids

Diterpenoid alkaloids can be isolated from the plants in the genera *Aconitum* and *Delphinium*. Most of the diterpenoid alkaloids have biological activities [3]. Therefore, they have great importance in drug development. There are more than 550 different *Aconitum* and *Delphinium* species in the world. Meanwhile, more than 300 diterpenoid alkaloids have been identified. The diterpenoid alkaloids may be divided into two broad categories: (a) with a C_{19}-skeleton and (b) with a C_{20}-skeleton. The C_{19}-diterpenoid alkaloids, commonly called aconitines, may further be divided in four groups: aconitine-type, lycoctonine-type, pyrodelphinine-type, and heteratisine-type [4].

1.1.3 Aconitine-type alkaloids

At present, there are more than 50 known aconitine-type alkaloids in literature [5]. In the present work, only hypaconitine (HA), mesaconitine (MA), and aconitine (A) were studied (see Figure 1.1). They are bioactive ingredients in the roots of the plants *Aconitum carmichaeli* and *Aconitum kusnezoffii* [6,7]. The processed parent root of *Aconitum carmichaeli* and root tuber of *Aconitum kusnezoffii* are known as "Chuanwu" and "Caowu", respectively, and are used in Chinese herbal medicinal practice to relieve rheumatic conditions and alleviate pain [8]. HA, MA, and A have analgesic, antipyretic, anti-inflammatory, cardiotonic, and local anesthetic properties [9,10] but are highly toxic. They have formally been used as an arrow poison in hunting and in war [11]. They are found to exert harmful effects on the cardial contractive function in animals [12], and can cause arrhythmia [13]. There is a very narrow margin of safety between a therapeutic and toxic dose.



Compounds	R_1	R_2	MW
Hypaconitine	CH_3	H	615
Mesaconitine	CH_3	OH	631
Aconitine	C_2H_5	OH	645

Figure 1.1 Structure and molecular weight (MW) of aconitine-type alkaloids

1.1.4 Toxicity

From 1989 to 1995, the public hospitals in Hong Kong have managed more than thirty cases of herb-induced aconite poisonings [14,15]. Three of them were fatal cases [16,17]. The situation was even worse in China, where two thousand six hundred and four cases of aconite poisoning have been documented in the period from 1958 to 1992. Thrity-nine of them were fatal cases [18]. The actual number of aconite poisoning cases might be much higher due to the difficulties in the identification of aconite poisoning.

Severe poisoning has been reported after ingestion of as little as 0.2 mg aconitine [19]. As a result, aconite roots are commonly labeled as potent herbs and their usage strictly controlled in China and Taiwan (Table1.1). Only qualified practitioners and dispensers are allowed to prescribe or handle these potent herbs. There is, however, no legislative control of these potent herbs in Hong Kong [20].

1.1.5 Safety concerns

Although all *Aconitum* roots used for medical purposes contain highly toxic aconitine-type alkaloids, substantial variations in toxicity have been observed due to qualitative and quantitative variations of these alkaloids. These variations depend on the organs of the plants [21], plant species [22], and other seasonal factors [23]. In addition, the toxicity of *Aconitum* roots can be greatly reduced by heating, steaming and soaking [24-26]. Throhgh these processes, the highly poisonous aconitine-type alkaloids can be hydrolyzed to the much less toxic benzoylaconine and aconine analogues [27]. Figure 1.2 shows a schematic diagram of the hydrolysis of aconitine.

The toxicities of the unhydrolyzed and hydrolyzed alkaloids in mice are shown in Table 1.2 [3]. It is, however, noted that the aforesaid processes of these *Aconitum* roots have not been standardized and may not be perfomed properly. The composition of the alkaloids and hence the toxicity and/or the therapeutic effectiveness of processed

Table 1.1

Potent or Toxic Chinese Medicinal Materials Controlled in China and Taiwan

Herbs	Controlled in
Root-tuber of *Aconitum brachypodum*	China
Root of *Aconitum carmichaeli*	Taiwan China
Unprocessed root of *Aconitum carmichaeli*	Taiwan China
Unprocessed lateral root of *Aconitum carmichaeli*	China
Root of *Aconitum coreanum*	Taiwan
Root of *Aconitum kusnezoffii*	Taiwan China
Unprocessed root of *Aconitum kusnezoffii*	Taiwan China

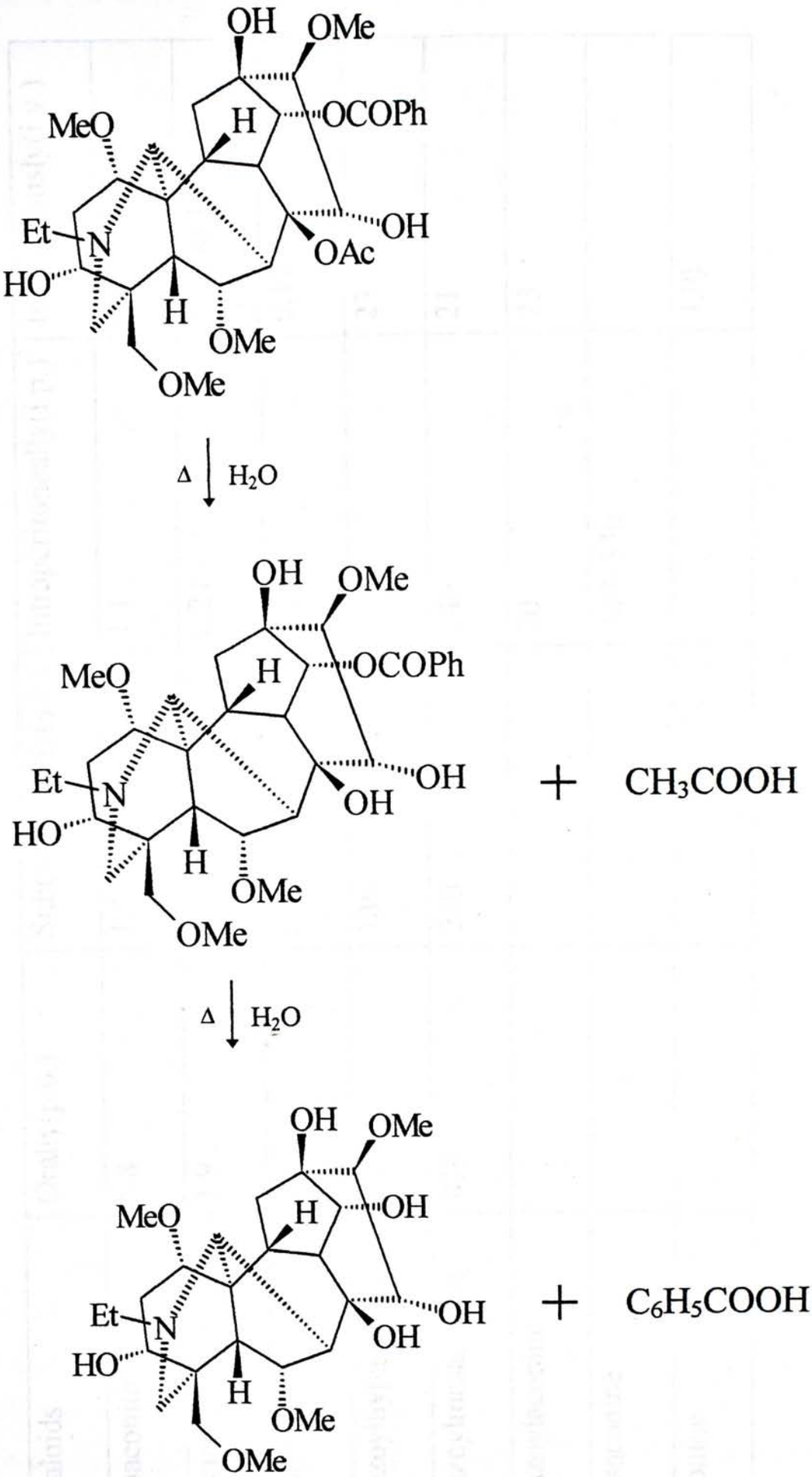


Figure 1.2 Schematic diagram of hydrolysis of aconitine

Table 1.2

LD_{50} of aconitine-type alkaloids and their hydrolysis products in mice (LD_{50}, mg/kg) [3]

Alkaloids	Orally(p.o.)	Subcutaneously(s.c.)	Intraperitoneally(i.p.)	Intravenously(i.v.)
Hypaconitine	5.8	1.2	1.1	0.47
Mesaconitine	1.9	0.20~0.26	0.21	0.10~0.13
Aconitine	1.8	0.27~0.31	0.31	0.12
Benzoylhypaconine	830	130	120	23
Benzoylmesaconine	810	230	240	21
Benzoylaconine			70	23
Mesaconine			300~330	
Aconine				120

Aconitum roots varies substantially from one sample to another. Because of the life-threatening consequences, there is a pressing need to develop highly sensitive analytical methods to identify and quantify the potent constituents, such as aconitine, hypaconitine and mesaconitine, in the raw and processed *Aconitum* roots.

1.2 Summary of Previous Work

The aconitine-type alkaloids in Aconitum roots have been examined by a number of analytical methods. Attempts to quantify the active ingredients in natural products by using conventional spectroscopic methods have always been hampered by the complex sample matrices which lead to strong signal interferences. Early attempts to characterize the alkaloid contents in *Aconitum* roots have been carried out by using spectrophotometric methods [28-30]. In order to alleviate the problems of chemical interference, Xiong and co-workers [30] utilized bromothymol blue as a reagent to bind specifically with aconitine-type alkaloids in a buffered solution (pH ~6) to form stable ion-pair complexes. The amount of aconitine-type alkaloids were then evaluated by measuring the absorption coefficients of the ion-pair complexes at 410 nm. Alternatively, Wu et al. [31] has demonstrated the use of aconitine-selective membrane electrode to determine the content of aconitine in samples by electrochemical methods. In spite of the high selectivity, these methods can only provide an overview of the amount of aconitine-type alkaloids. Detailed distribution of various alkaloid species cannot be obtained. In addition, typical sensitivity is only in the order of sub-milligrams per gram of samples.

To avoid tedious isolation and purification procedures, chromatographic techniques have been used in conjunction with spectroscopic analysis to provide important information on the amount of various alkaloids in aconite samples. For instance, thin-layer chromatography (TLC) has been used in conjunction with densitometry to provide a simple and fast separation; and to quantify various main alkaloids in aconite roots [32,33]. Despite of the simplicity, the TLC method has a rather low sensitivity and a limited separation efficiency. High performance liquid chromatography (HPLC) [34-38]

has shown to provide better separation and a lower limit of detection. With a UV detector, a detection limit (signal-to-noise ratio, 3) of low nanograms was demonstrated. Since identification of alkaloids by using chromatographic retention time can easily be interfered by the presence of other constituents having similar elution properties, the results from one experiment must be substantiated by chromatography using a different separation mode such as ion-pair chromatography. Even with two different chromatographic separation methods, the identification remains indirect and is likely to be affected by the presence of other chemical constituents. In addition, chromatographic separation is normally tedious and time-consuming. The sensitivity depends also on the absorption coefficients of the alkaloids in the selected UV wavelength.

The use of mass selective detector, such as quadrupole mass spectrometer, provides an online identification of the chromatographic peaks and prevents any accidental co-elution of two or more constituents which would otherwise lead to substantial error in quantification. While mass spectrometry remains one of the very sensitive analytical methods, replacement of the conventional UV detetctor by mass spectrometer would also lead to a substantial increase in the sensitivity of the measurement and hence reducing the detection limit.

In order to use mass spectrometry as a mass selective detector for chromatography (or to use chromatrographic techniques as pre-purification tools for mass spectrometry), the intrinsic problems of interfacing two distinctively different techniques will need to be solved. Interfacing gas chromatography to mass spectrometry is relatively simple due to the low flow rate of the carrier gas. Direct insertion of the chromatographic column into the ion source region of the mass spectrometer is possible. Gas chromatography-mass spectrometry (GC/MS) is ideal for the simultaneous separation and identification of volatile and/or thermally stable compounds in complex mixture. The necessity of vaporizing all constituents before the chromatographic separation has, however, imposed substantial constraints onto the classes of compounds amenable to the analysis. For instance, aconitine-type alkaloids cannot be analysed by GC/MS without prior

derivatization. Ito and co-workers [23,39] have investigated the feasibility of using gas chromatography with mass spectrometer detector for the analysis of aconitine-type alkaloids. To increase the volatility and thermal lability of alkaloids, trimethylsilyl (TMS) derivatization of the alkaloids was found to be essential prior to the GC/MS analysis. This method has successfully been used to separate and quantify aconitine-type alkaloids in both *Aconitum* root and in human serum. By using selected ion monitoring (SIM) technique, good linear response over the range of 100 pg to 7.5 ng was demonstrated for various alkaloids investigated.

To separate involatile and thermally labile compounds, high performance liquid chromatography-mass spectrometry is usually prefered. It is, however, more difficult to interface the high flow of liquid (i.e. millilitres per minute) from the outlet of the HPLC to the mass spectrometer without quenching the high vacuum conditions of the spectrometer. Several approaches have been developed to overcome this difficulty. Examples include continuous-flow fast atom bombardment (FAB), particle beam (PB) technique and thermospray (TSP). In continuous-flow FAB, sample liquid is pumped through a special designed sample probe in which the front end of the probe contains a semi-permeable "frit". Only a small portion of the liquid sample can penetrate into the outer region (i.e. the high vacuum side) of the probe where it is desorbed and ionized by a beam of kiloelectron volt atomic beam [40]. Alternatively, PB was originally developed as a 'monodisperse aerosol generating interface for chromatography' (MAGIC) by Willoughby and Browner [41]. In PB interface, the column effluent is nebulized into a near atmospheric-pressure desolvation chamber which is connected to a momentum separator where the high molecular-weight analytes are preferentially transferred to the MS ion source. The analyte molecules are transferred in small particles to a conventional EI/CI ion source, where they disintegrate by hitting a heated target and the released molecules are ionized by EI or CI. In TSP interface, a jet of vapour is formed out of a heated vaporizer tube. Nebulization takes place as a result of the disruption of the liquid by the expanding vapour that is formed upon evaporation of part of the liquid in the tube. By applying efficient pumping directly at the ion source, aqueous solvents (with flowrate

up to 2 mL/min) can be introduced into the MS vacuum system. The ionization of the analytes takes place by means of chemical ionization reactions and ion evaporation processes [42].

In the past, liquid chromatography-mass spectrometry (LC/MS) has not received much attention due partly to the complexity of the techniques and the instrumentation involved and partly to the low efficiency of the interface. Typical sensitivity of these techniques is in the range of nanomoles. More recently, the development of atmospheric pressure ionization techniques (including atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI)) has aroused public interest in the LC/MS. APCI is a soft ionization (less fragmentation) technique. In an APCI ion source, a high voltage on a pointed electrode at atmospheric pressure creates a corona discharge and generates many electrons. These electrons interact with nitrogen and oxygen gases to form positively charged ions. These ions then undergo charge exchange reactions with water vapor in the ion source to form hydronium ions. Finally gaseous analyte molecules react with the hydronium ions to form the corresponding ions. Figure 1.3 shows a schematic diagram of the fundamental processes for APCI. APCI is typically used to analyze compounds of medium polarity that have some sort of volatility. It can accommodate flow rates of sub-microlitre to millilitre per minute. Electrospray ionization is the softest ionization technique currently available. It transforms analyte ions from solution into gas phase. Although the mechanism of ion formation in ESI remains controversial, there are several commonly accepted processes. As solvent and solute exit electrospray needle, a high-voltage gradient at the tip of the needle disrupts the liquid surface and produces a fine mist of droplets. These droplets are electrically charged at their surfaces. The charge density at the surface of the droplets increases as solvent evaporates and the size of the droplet shrinks. At a critical point, known as the Rayleigh stability limit, the charge density is so high that the droplet divides into smaller droplets due to Coulombic repulsion. The process is repeated many times to form very small droplets. From the very small, highly charged droplets, sample ions are ejected into the gas phase [44]. The ions formed then enter the mass spectrometer and are mass-analyzed. Figure 1.4 shows a

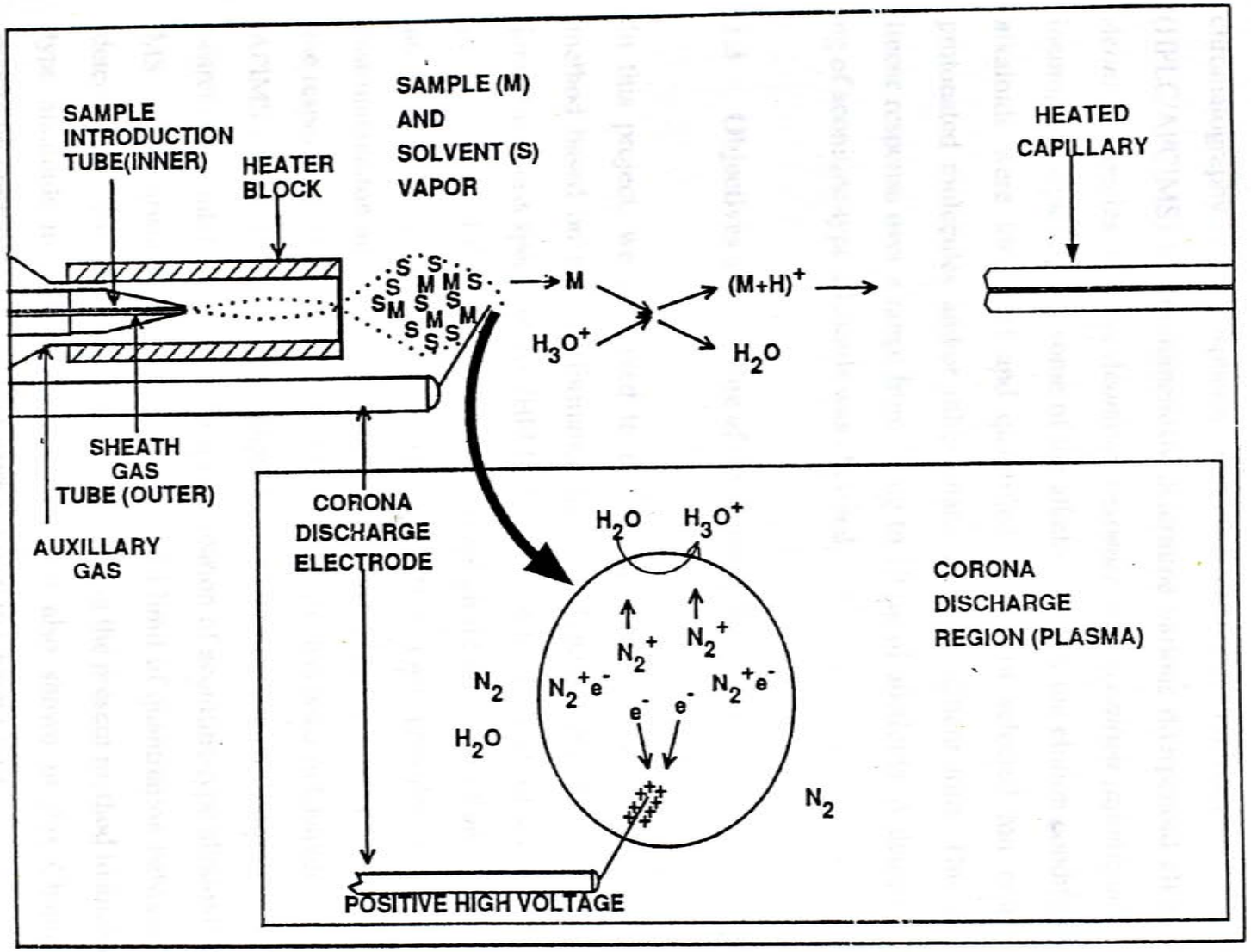


Figure 1.3 Basic process for atmospheric pressure chemical ionization; inset shows primary and secondary ion formation in the corona discharge region [43]

schematic diagram of the important processes in electrospray ionization. Electrospray ionization can be used to analyze polar compounds that have essentially no volatility. It can accommodate flow rates of 1 μL/min to 1 mL/min.

Wada and co-workers [45, 46] have reported the use of high performance liquid chromatography / atmospheric pressure chemical ionization mass spectrometry (HPLC/APCIMS) to simultaneously determine various diterpenoid alkaloids in several *Aconitum* species, such as *Aconitum yesoense* and *Aconitum japonicum*. Despite of the incomplete separation of some of the alkaloids under the elution conditions reported, all alkaloids were identified and quantified by use of selected ion monitoring on the protonated molecules and/or other characteristic fragment ions. This method showed linear response over a range from 10 ng to 10 μg of alkaloids. A detection limit of 2-20 ng of aconitine-type alkaloids was obtained.

1.3 Objectives and Outline of the Present Work

In this project, we attempted to develop a highly selective and sensitive analytical method based on high performance liquid chromatography and atmospheric pressure ionization mass spectrometry (HPLC/APIMS) for the analysis of aconitine-type alkaloids in Chuanwu and Caowu. Chapter One gives an introduction of aconitine-type alkaloids and a summary of previous works. Chapter Two provides an overview of the instrumentation and experimental methods. The effects of mobile phase composition on the response of the analytes in HPLC and MS are discussed in Chapter Three. Optimized APIMS conditions are also presented in this Chapter. Chapter Four shows the results of searching an internal standard for quantification of aconitine-type alkaloids in HPLC and MS. A comparison of the detection limit, and limit of quantitation between UV and MS detectors are also presented. The results of using the present method to analyze aconitine-type alkaloids in Chuanwu and Caowu are also shown in this Chapter. Finally, a concluding remark of the present work is given in Chapter Five.

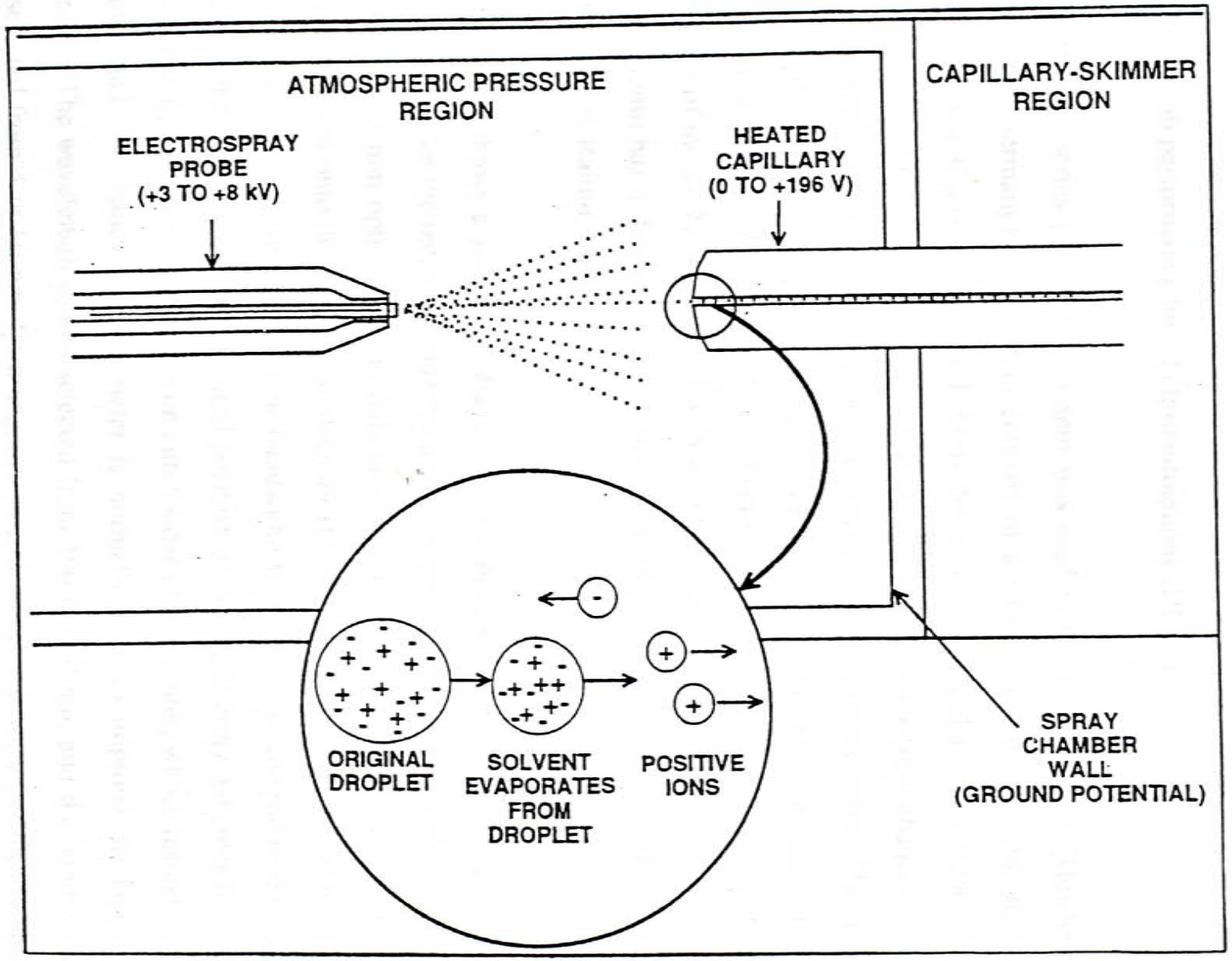


Figure 1.4 Schematic drawing of electrospray ionization that shows the formation of a spray of highly charged sample droplets [43]

CHAPTER TWO

INSTRUMENTATION AND EXPERIMENTAL

2.1 Instrumentation

2.1.1 High performance liquid chromatography (HPLC)

A HP1090 Series II/M HPLC system was used in the present study (Hewlett-Packard company, Germany). The system consists of a solvent delivery system, an injector, a reverse-phase C_{18} column, a diode-array detector (DAD) and a data system. The solvent delivery system equipped with three solvent channels. Each solvent channel comprises a solvent reservoir and a dual-syringe metering pump with rotary valve. The solvents are mixed in the low pressure compliance before being compressed to high pressure and pumped through a damping unit, an injector and into the C_{18} column. A Rheodyne™ injector of model 7010 equipped with six-port rotary valve was used. The reverse-phase C_{18} column has a dimension of 4.6 mm ID x 25 cm L and packed with 5 μm sorbates (Microsorb, Rainin, Massachusetts, U.S.A.).

Figure 2.1 shows a schematic diagram of the optical system of the DAD. The optical system focuses the incident light onto a 6 mm flow cell. The transmitted light then passes through a 2 mm optical slit and falls onto a holographic grating. The light is dispersed and reflected onto the diode-array detector (DAD). There are four important parameters in DAD, i.e. the wavelength and the bandwidth for the analyte and reference signals. The wavelength value defines the central position of the diode array, whereas the bandwidth value defines the range of diodes on either sides of this center, whose responses are to be averaged. The bandwidth parameter is normally used to improve the signal-to-noise ratios. The wavelength can be selected from 190 to 600 nm; and the bandwidth can be selected from 4 to 80 nm. Data acquisition and processing were performed on a HPLC Chemstation (Pascal Series) using an operating software version 4.22.

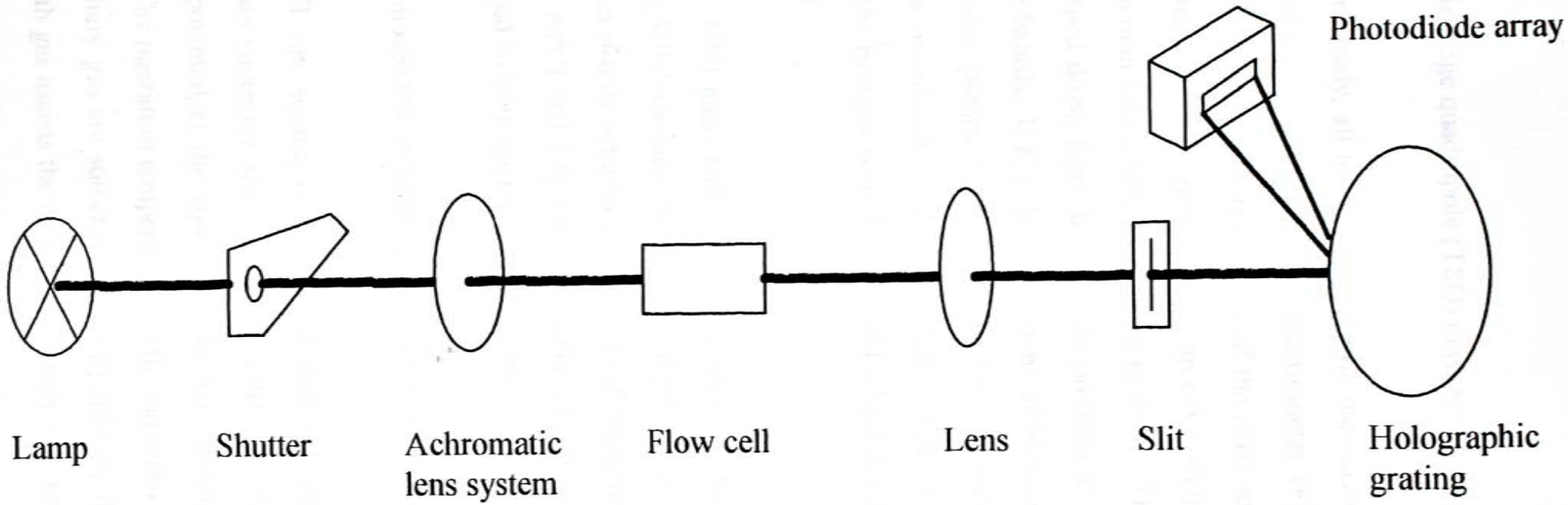


Figure 2.1 DAD light path

2.1.2 Triple-stage quadrupole (TSQ) mass spectrometer

In the present study, all mass spectrometric measurements were carried out with a TSQ 7000 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, U.S.A.). Figure 2.2 shows a schematic diagram of the mass spectrometer. Basically, it comprises of an ion source, an analyzer assembly, an ion detection system and a vacuum assembly. The vacuum manifold is normally heated to 70 °C. The pressure of the vacuum assembly is first pumped down from atmospheric pressure to ~10^{-2} Torr by using a rotary pump (E2M30, Edwards, U.K.). High vacuum conditions are achieved with two 250 L/s turbomolecular pumps (Turbo V-250, Varian, Italy). The vacuum condition of the manifold is monitored by an ion gauge (Type 563 Ionization Gauge, Varian, Italy) located at the position near detector and a base pressure of ~2 x 10^{-6} Torr are normally maintained.

The TSQ 7000 mass spectrometer is equipped with a standard atmospheric pressure ionization (API) interface. This interface comprises of a heated capillary, a tube lens, a skimmer, an rf-only octapole and a series of electrodes (L1 lenses) and is shown in Figure 2.3. Since APCI and ESI share the same interface, these ionization techniques can be interchanged without breaking the vacuum system.

2.1.2.1 Atmospheric pressure chemical ionization (APCI)

The APCI ion source consists of a stainless steel sample inlet assembly, a high temperature vaporizer and a corona needle. Manipulation of the vaporizer temperature and the potential at the corona needle are important for the formation of stable ion signals. The operation temperature for the vaporizer is roughly 400 to 600°C. Sheath gas and auxiliary gas are sometimes used to enhance the performance of the APCI source. The sheath gas assists the droplet formation, whereas the auxiliary gas helps to focus the

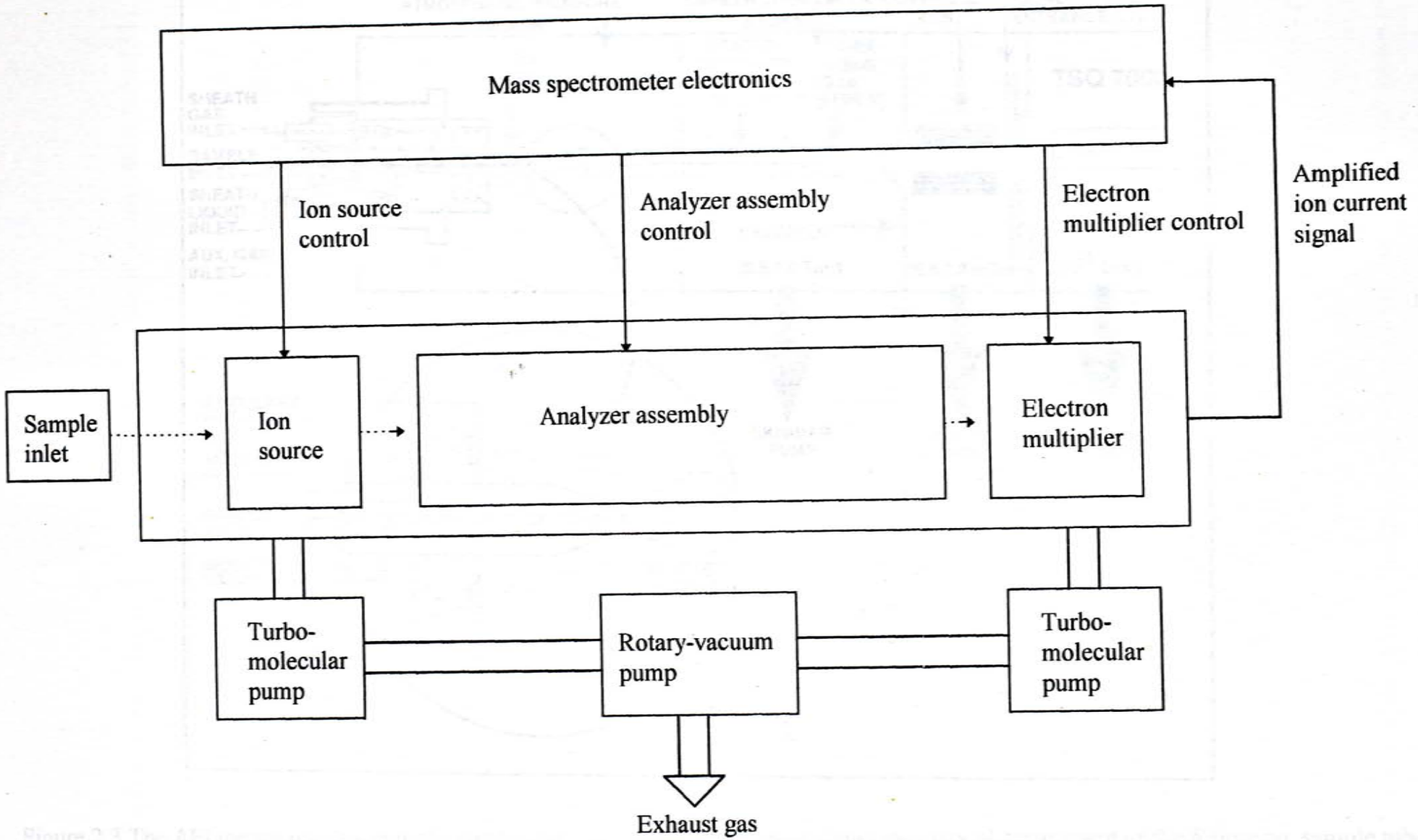


Figure 2.2 Block diagram for the major components of a mass spectrometer

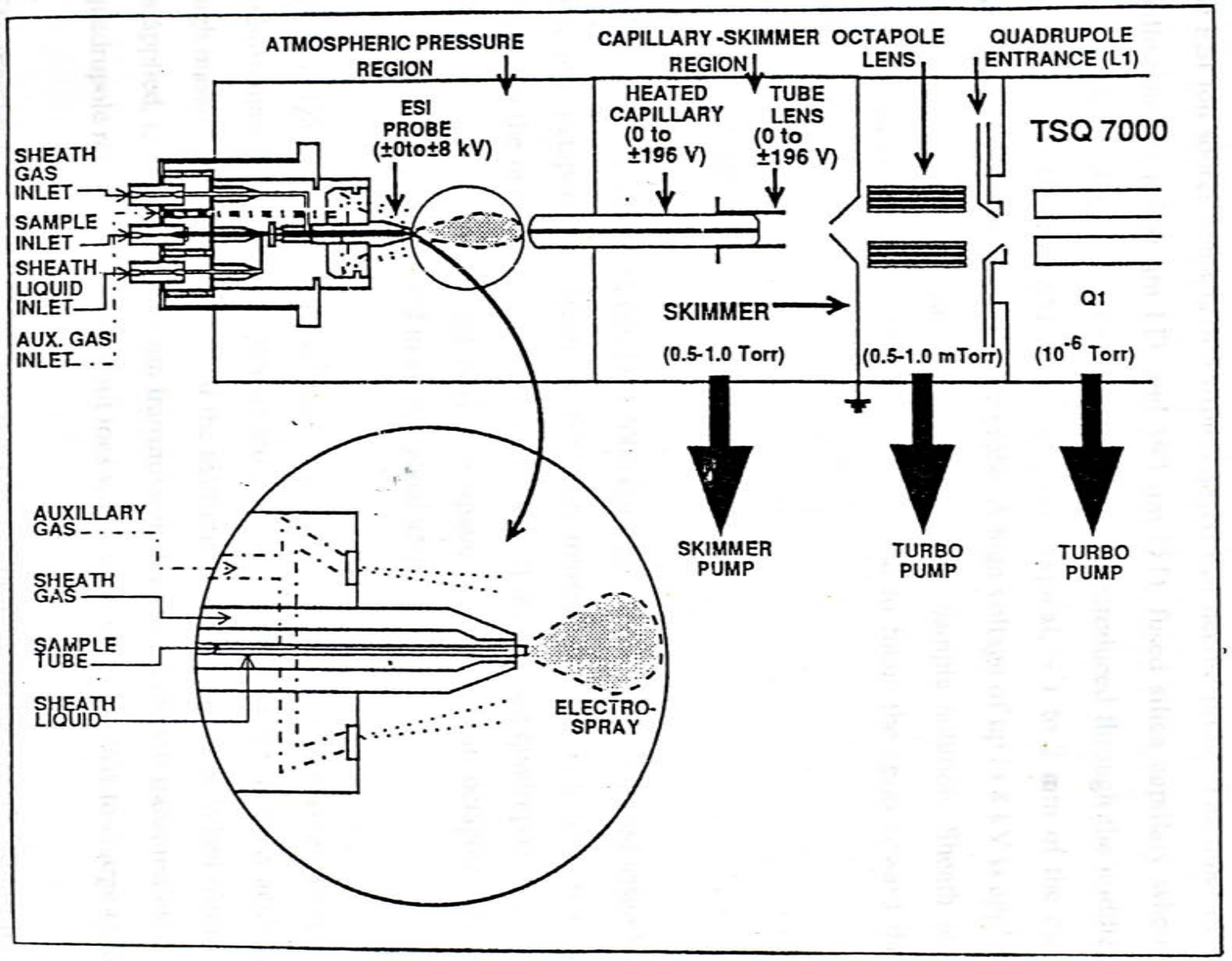


Figure 2.3 The API ion source shown in the electrospray ionization mode; inset shows the coaxial arrangment of the following: sample tube, sheath liquid tube (electrospray needle), sheath gas tube, and auxiliary gas outlet ports [43]

spray toward the heated capillary and helps to keep spray chamber dry. The maximum flow for sheath gas and auxiliary gas are 80 psi and 65 (arbitrary units), respectively.

2.1.2.2 Electrospray ionization (ESI)

The ESI ion source consists of a triple-layered ESI needle probe. The inner layer of the needle-probe is a 100 μm I.D. and 190 μm O.D. fused silica capillary where analyte solution flows. Sheath gas and auxiliary gas are introduced through the middle and the outer layers to stabilize and assist the spray. Typical, ~ 1 to 2 mm of the capillary is protruded from the tip of the needle probe. A high voltage of up to 8 kV is applied to the ESI needle probe to initiate the spraying of the sample solution. Sheath gas and/or auxiliary gas are used to stabilize the spray and to focus the spray toward the heated capillary.

2.1.2.3 Quadrupole system

The analyzer assembly on the TSQ 7000 consists of two quadrupole rod assemblies (Q1, Q3), one octapole rod assembly (Q2 ion transmission device), and four lens sets (including the one in the source) (Figure 2.4). Q1 and Q3 are quadrupole rod assemblies composed of four metal rods held in a square array. Q2 is an octapole rod assembly composed of eight rods held in an octagonal array.

Q1 and Q3 can either act as mass analyzers or as ion transmission devices. When radiofrequency (rf) and dc voltages are applied, Q1 and Q3 act as mass analyzers. The high mass-to-charge (m/z) limit of the instrument is 4,000 Daltons. When rf-only voltage is applied, Q1 and Q3 act as ion transmission devices. In the ion transmission mode, the quadrupole rod assemblies transmit ions with a wide range of mass-to-charge ratios.

Q2 operates with a rf-only voltage to act as an ion-focusing device. By admitting an inert gas (typically argon) to a pressure of 0.8 mTorr to 4 mTorr, collision-induced

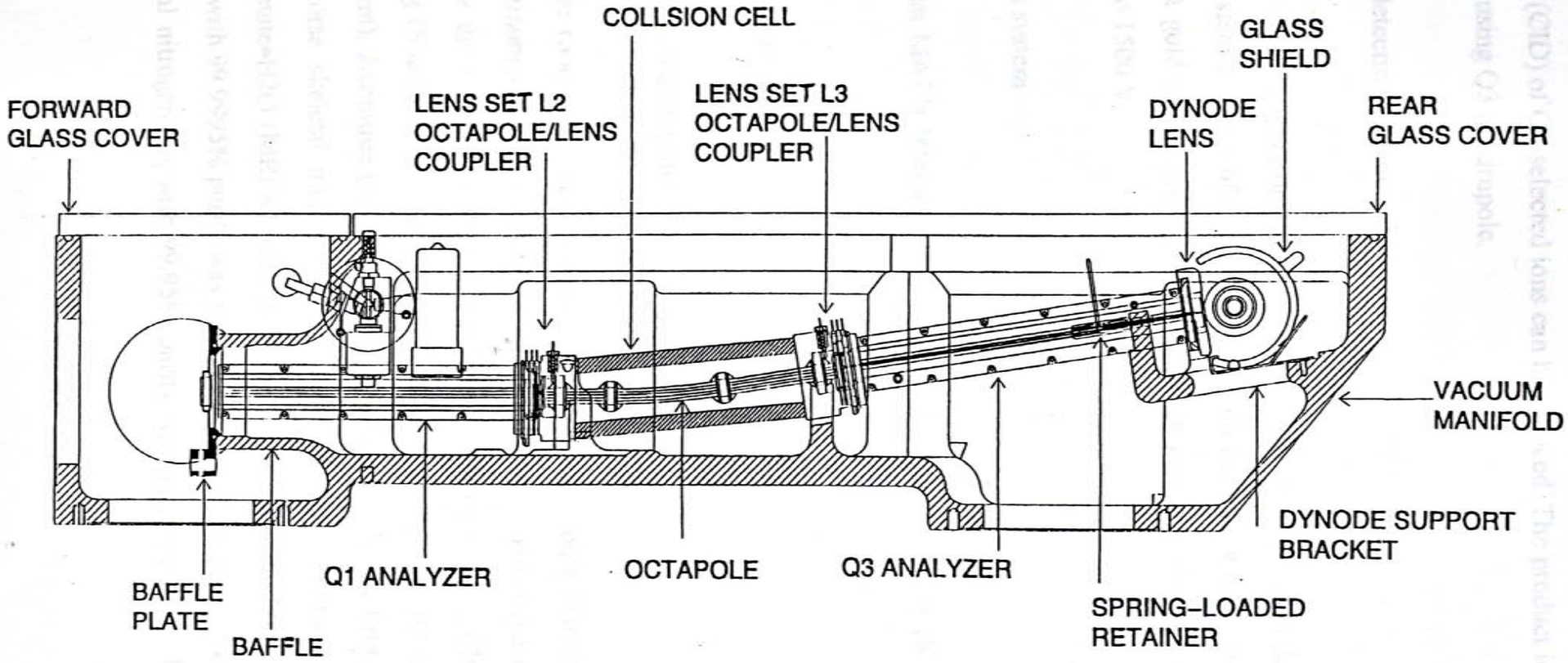


Figure 2.4 The analyzer assembly, showing the position of the lenses [47]

dissociation (CID) of Q1-selected ions can be induced. The product ions formed can then be analyzed using Q3 quadrupole.

2.1.2.4 Ion detection system

The TSQ 7000 mass spectrometer is equipped with an off-axis ion detection system. The detection system consists of a 15 kV conversion dynode, a continuous-dynode electron multiplier, a gold-plated glass ground shield. In practice, electron multiplier may vary from 1000 to 1500 V.

2.1.2.5 Data system

The Finnigan MAT's Interactive Chemical Information System (ICIS^{TM}) software was used.

2.2 Experimental

2.2.1 Sample and reagents

Raw aconite roots (Chuanwu, Caowu), cured aconite roots (Zhichuanwu, Zhicaowu), dimethylaminobenzaldehyde (DAB), psoralen (PSO), and hypaconitine (HA) were supplied by the Chinese Medicinal Material Research Centre, Chinese University of Hong Kong (Shatin, H.K.). Mesaconitine (MA) with 98% purity was purchased from Wako (Japan). Aconitine (A) with 95% purity was purchased from Sigma (St. Louise, U.S.A.). Horse skeletal muscle apomyoglobin and L-methionyl-arginyl-phenylalanyl-alanine acetate•H2O (MRFA) were purchased from Finnigan MAT (San Jose, U.S.A.). Argon gas with 99.9995% purity was purchased from Hong Kong Special Gas Co. (H.K.). Commercial nitrogen gas with 99.95% purity was purchased from Hong Kong Oxygen (H.K.).

Acetonitrile (ACN) and tetrahydrofuran (THF) of HPLC grade, and diethyl ether of AR grade were purchased from Mallinckrodt (Kentucky, U.S.A.). Triethylamine (TEA) of GC grade, and acetic acid glacial 100% of GR grade were purchased from Merck (Frankfurter, Germany). Ammonium acetate of AR grade was purchased from Peking' s reagent (Peking, P.R.C.).

2.2.2 Sample preparation

Figure 2.5 shows the procedure used for extraction of alkaloid contents from aconite roots. The finely grinded aconite roots were filtered by 60 mesh sieve. 400 μg of the powder sample was carefully weighted and placed into a 25 mL Erlenmeyer flask. 0.4 mL 33 % ammonia solution was added. The solution was allowed to stand for 2 hours [35]. The solution was filtered with a funnel containing a piece of cotton wool. The solid residues were rinsed successively with 2 mL diethyl ether for three times. The filtrates were combined and evaporated to dryness. The solid deposit was re-dissolved with 1 mL acetonitrile. The resulting solution was filtered with 0.45 μm filter before HPLC/APIMS analysis.

2.2.3 High performance liquid chromatography conditions

In the present study, the mobile phase contained three components, namely 'A', 'B' and 'C', in a volume ratio of 77:13:10. 'A' was an aqueous buffer solution containing 0.5% triethylamine (TEA), 50 mM ammonium acetate and 1 % acetic acid. 'B' and 'C' were pure acetonitrile and tetrahydrofuran (THF), respectively. The flow rate was maintained at 0.8 mL/min. Drawing speed of the injector was 83 μL/min and the injection volume was 5 μL. Wavelength of signal and reference were set at 230 and 450 nm respectively. Both signal and reference bandwidths were set at 4 nm.

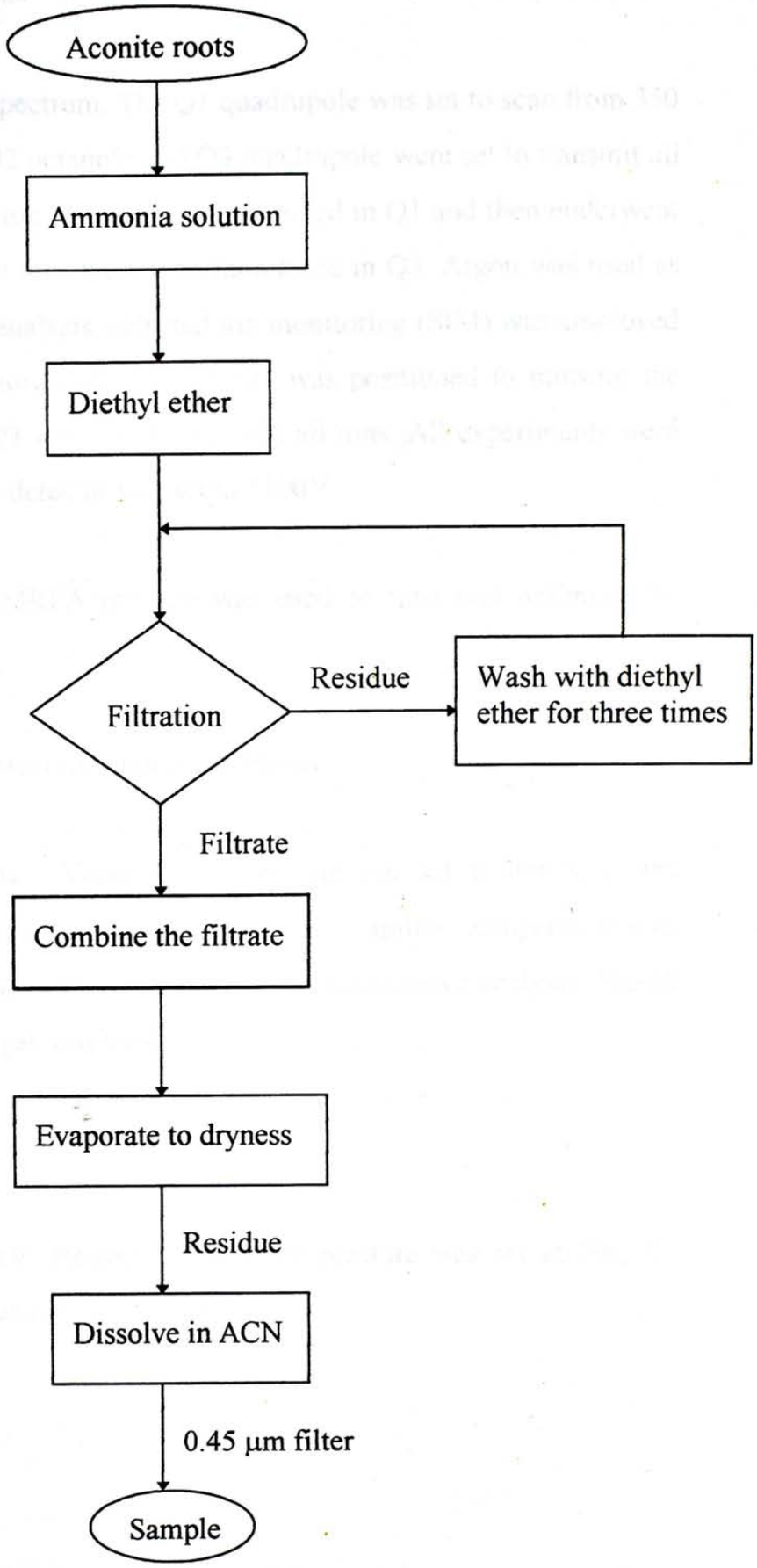


Figure 2.5 Flow chart of extraction

2.2.4 Mass spectrometry conditions

In the acquisition of normal mass spectrum, The Q1 quadrupole was set to scan from 350 to 750 amu at 1 second, whereas Q2 octapole and Q3 quadrupole were set to transmit all ions. In product ion scan mode, an ion of interest was selected in Q1 and then underwent fragmentation in Q2. The fragment ions were then monitored in Q3. Argon was used as the collision gas. For quantitative analysis, selected ion monitoring (SIM) was employed to enhance the sensitivity of measurement. In SIM, Q1 was positioned to transmit the ions of interest, whereas Q2 and Q3 were set to transmit all ions. All experiments were operated at positive-ion mode. The detector was set to 1100V.

Standard solutions of myoglobin/MRFA mixture was used to tune and optimize the conditions of the instrument.

2.2.4.1 Atmospheric pressure chemical ionization conditions

The corona current was set at 5 μA. Vaporizer temperature was set at 500 °C in the qualitative analysis, and 400 °C in the quantitative analysis. Capillary temperature was set at 200 °C in the qualitative analysis, and 135 °C in the quantitative analysis. Sheath gas was set at 40 psi. No auxiliary gas was used.

2.2.4.2 Electrospray ionization conditions

The spray voltage was set at 5 kV. Heated capillary temperature was set at 200 °C. Sheath gas was set at 40 psi. No auxiliary gas was used.

CHAPTER THREE

SELECTION AND OPTIMIZATION OF HPLC/MS METHOD

3.1 Introduction

It is well known that mobile phase composition exerts prominent effect on the elution of analytes in typical HPLC analysis. Through proper selection of the mobile phase composition, separation of analytes from one another and from the sample matrix can be optimized with minimal elution time. Apart from the separation efficiency, the mobile phase composition might also influence the ionization efficiency, the stability of API signals [48] and hence the sensitivity and reproducibility of the HPLC-MS method.

In this chapter, the effects of mobile phase composition on separation efficiency, elution time, mass spectrometry signals were investigated. The separation efficiency is determined by calculating the resolution (R):

$$R=2[(t_{RB}-t_{RA})/(W_A+W_B)]$$

or

$$R=1.176[(t_{RB}-t_{RA})/(W_{1/2A}+W_{1/2B})]$$

where t_{RB} and t_{RA} are the retention times; W_A and W_B are the peak width (PW) at base position; $W_{1/2A}$ and $W_{1/2B}$ are the PW at half height; of the two adjacent peaks. The acceptable values of R should be larger than 1.5 [8] such that the degree of peak overlap is less than 1 % [49].

Selection of elution conditions has been a challenging work because of the additional consideration on mass spectrometry interface. Apart from the chromatographic separation of the analytes, the elution conditions must also support either APCI and/or ESI processes. In the present experiment, reverse-phase chromatography was chosen to

separate aconitine-type alkaloids. The mobile phase was 77% water, 13% acetonitrile, and 10% tetrarhydrofuran. Apart from the good solubilities of standard aconitine alkaloids in this solution mixture, the resulting alkaloid solutions were found to produce stable signals in both APCI and ESI techniques.

In the analysis of basic substances such as the aconitine-type alkaloids using a reversed-phase HPLC system of chemically bonded silica gel, it is well known that tailing peaks are obtained due to the undesirable retention of the analytes by free silanol groups. Addition of long alkyl chain amines in low concentrations to the mobile phase has been reported to improve peak shape in the HPLC analysis of basic compounds [50]. Among different amines, the water-soluble triethylamine was found to be the best modifier for reversed-phase chromatography of basic substance [51]. However, silica dissolves readily in aqueous solvents at a pH higher than 8-9. The use of triethylamine solution would lead to the dissolution of the silica gel and hence column breeding [52]. Eventually, there would be a substantial pressure drop and the column efficiency would be much reduced. With regard to this limitation, an amine-phosphate buffer with pH of 2 to 3 [50,53] was investigated as eluent for separation of basic substances. Using phosphate buffer, Hikino et al [34] has succeeded in separating various alkaloids. It is, however, noted that the involatile phosphate salts would deposit inside the capillary tube at the HPLC-MS interface and block the liquid flow. In order to overcome this difficulty, a more volatile buffer system, such as acetic acid / ammonium acetate has to be used.

In this project, the effect of triethylamine, acetic acid and ammonium acetate on the separation efficiency, elution time, mass spectrometry signals were investigated. Table 3.1 summarizes the parameters to be investigated. Effort has been made to develop an isocratic elution protocol rather than gradient elution so as to avoid re-equilibration of column conditions between successive experiments [54].

Table 3.1

Summary of the parameters to be optimized: (a) mobile phase composition, and (b) API inlet

	Parameters	Experimental setting				
(a)	TEA (%)	0	0.2	1	1.5	2
	Ammonium acetate (mM)	0	5	25	50	75
	Acetic acid (%)	0.2	1	2	3	
(b)	Corona current (μA)	3	4	5	6	7
	Vaporizer temperature (°C)	300	350	400	450	500
	Capillary temperature (°C)	125	135	150	175	200
	Auxiliary gas (arbitrary units)	0	5	10	20	
	Sheath gas (psi)	20	40	60	80	

3.2 Experimental

The sample used was a mixture consisted of 120 ppm HA, MA, and A. The buffer of the mobile phase was prepared by dissolving certain amount of ammonium acetate, acetic acid and TEA in ultra-pure water (18 mΩ).

In APCI mode, corona current was set at 5 μA. The temperature of vaporizer and heated capillary were set at 400 °C and 150 °C respectively. Meanwhile, the sheath gas and auxiliary gas were set at 40 psi and 10 arbitrary units respectively. In ESI mode, spray voltage was set at 5 kV. While the temperature was set at 200 °C. The sheath gas and auxiliary gas were set at 40 psi and 10 arbitrary units respectively. In the mass analyzer, SIM mode was used. The selected ions were 556, 572, 586, 616, 632, and 646. The scan time was set at 1 second.

3.3 Results and Discussion

In order to maximize the sensitivity and selectively of the on-line UV detector, the ultraviolet spectra of aconitine-type alkaloids standards were measured. Figure 3.1 shows a typical ultraviolet absorption spectrum of mesaconitine. As expected, all aconitine-type alkaloids of interest exhibited strong absorption at ultraviolet region and negligible absorption at near visible region. The strong absorption at 230 nm is consistent with the presence of a phenyl substitute (as shown in Figure 1.1) within the alkaloids. The wavelengths for signal and reference were therefore fixed at 230 nm and 450 nm. The bandwidths of signal and reference were found to have little influence on the quality of the chromatogram and were arbitrary set at 4 nm.

In the course of optimizating the mobile phase composition for separation and ionization of the target alkaloids, the effects of acetic acid, ammonium acetate and triethylamine were found to interrelated, i.e. their effects were not additive. Much effort was devoted to search for a composition that satisfied three important criteria, i.e. (i) good separation of

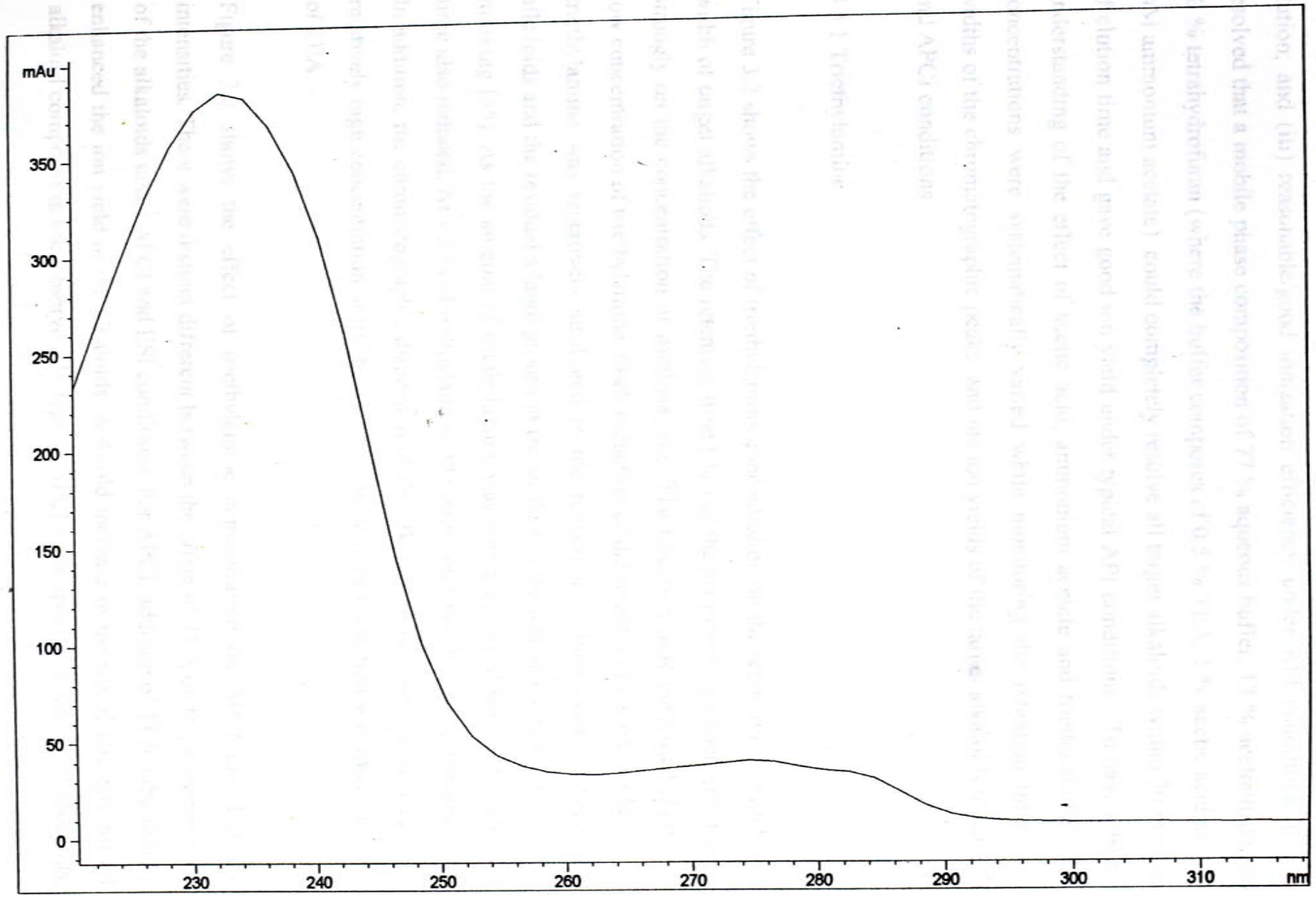


Figure 3.1 UV spectrum of MA (150 ppm) in ACN.

the target alkaloids from each other and from the residue matrix; (ii) minimal time of elution; and (iii) reasonable/good ionization efficiency under API conditions. It was resolved that a mobile phase composition of 77 % aqueous buffer, 13 % acetonitrile and 10 % tetrahydrofuran (where the buffer composes of 0.5 % TEA, 1 % acetic acid and 50 mM ammonium acetate) could completely resolve all target alkaloids within 30 minutes of elution time and gave good ion yield under typical API conditions. To have a better understanding of the effect of acetic acid, ammonium acetate and triethylamine, their concentrations were systematically varied while monitoring the retention times, the widths of the chromatographic peaks, and the ion yields of the target alkaloids under ESI and APCI conditions.

3.3.1 Triethylamine

Figure 3.2 shows the effect of triethylamine concentration on the retention time and peak width of target alkaliods. The retention time (t_R) of the aconitine-type alkaloids depend strongly on the concentration of triethylamine. The retention time decreased sharply at low concentration of triethylamine. Such reduction of the retention time upon addition of triethylamine was tentatively attributed to the reduction of interactions between the alkaloids and the residual silanol groups at the surface of the stationary phase; i.e. silanol masking [55]. As the amount of triethylamine was increased, its effect on the retention time also reduced. At 1.5 % of triethylamine, the retention time shows a minimum value. In addition, the chromatographic dispersion of the alkaloid signals were also reduced at relatively high concentration of triethylamine. A maximum resolution was found at 1.5% of TEA.

Figure 3.3 shows the effect of triethylamine concentration on APCI and ESI ion intensities. There were distinct different between the effect of TEA on the ion generation of the alkaloids under APCI and ESI conditions. For APCI, addition of TEA substantially enhanced the ion yield of the alkaloids. A 4-fold increase in the signal intensity for all alkaloid components was observed. In typical APCI mass spectra of the target alkaloids,

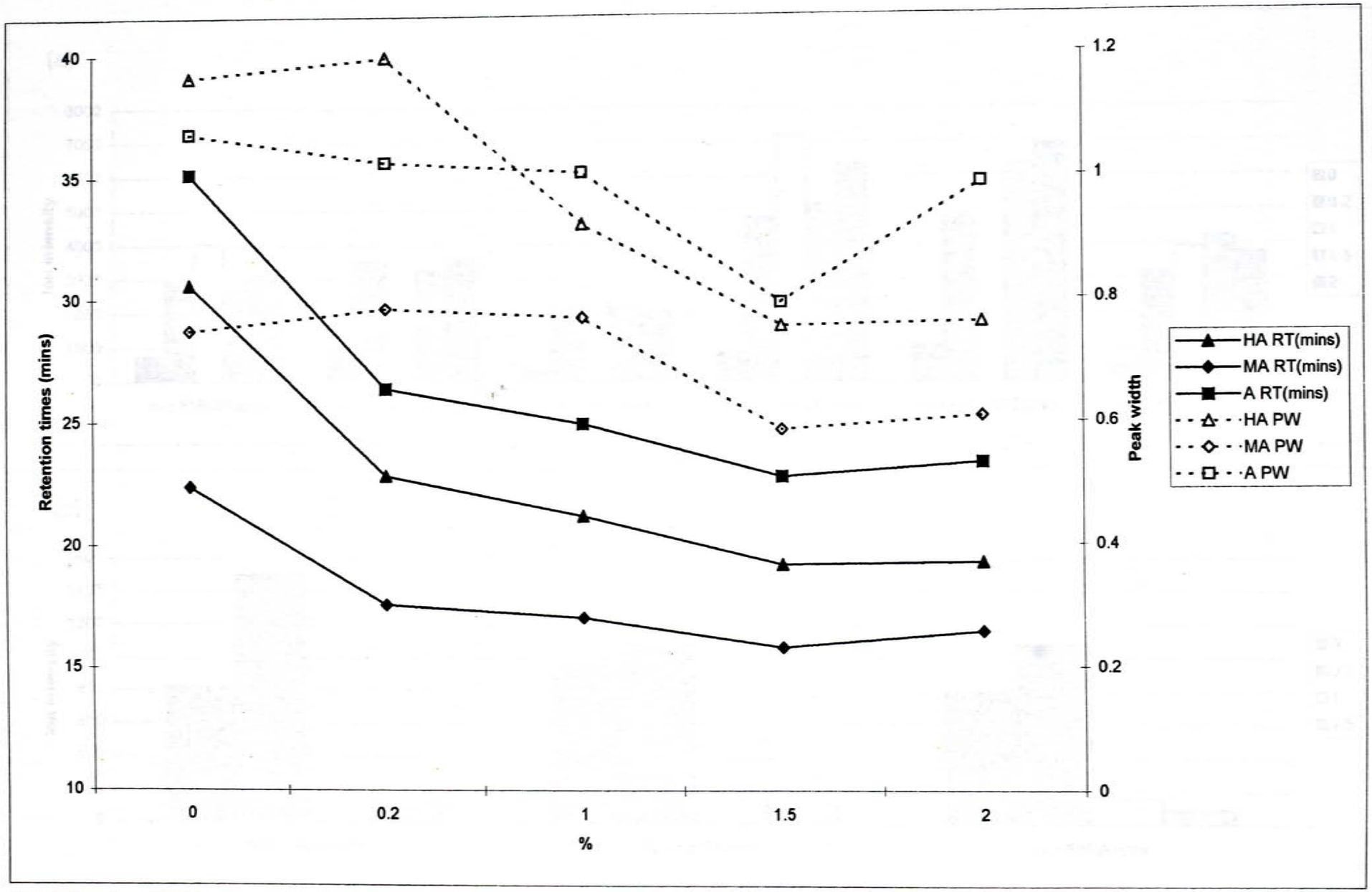


Figure 3.2 Effects of TEA concentration on retention times and peak width of aconitine-type alkaloids with 50 mM ammonium acetate and 1 % acetic acid

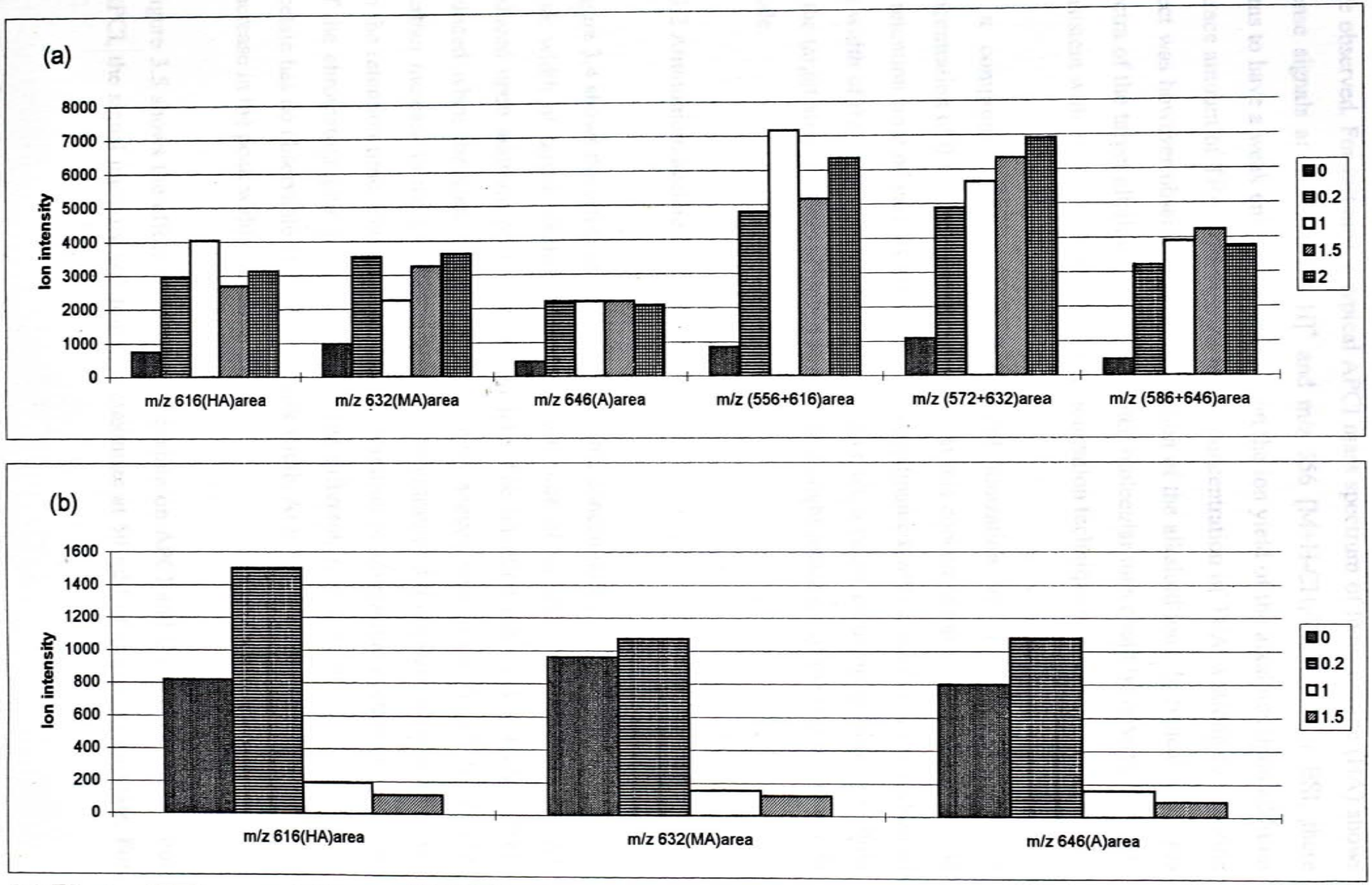


Figure 3.3 Effects of TEA concentration on: (a) APCI ion intensity and (b) ESI ion intensity of aconitine-type alkaloids with 50 mM ammonium acetate and 1 % acetic acid

both protonated molecular ions and the corresponding $[M+H-CH_3COOH]^+$ fragment ions were observed. For instance, a typical APCI mass spectrum of hypaconitine (HA) shows intense signals at m/z 616 $[M+H]^+$ and m/z 556 $[M+H-CH_3COOH]^+$. For ESI, there seems to have a weak enhancement effect on the ion yield of the alkaloids upon addition of trace amount of TEA (0.2 %). At higher concentration of TEA, a strong suppression effect was however observed for the formation of the alkaloid ions. In typical ESI mass spectra of the target alkaloids, only protonated molecular ions could be observed. This is consistent with the fact that ESI is a softer ionization technique than APCI.

As a compromise between separation and ionization of the target alkaloids, a concentration of 0.5 % of TEA was selected. At this concentration of TEA, the reduction of retention time of various alkaloids was accompanied with a substantial reduction of the width of the chromatographic peaks. As a result, a better chromatographic resolution of the target alkaloids was achieved in spite of a slight reduction of the sensitivity in ESI mode.

3.3.2 Ammonium acetate

Figure 3.4 shows the effect of ammonium acetate concentration on the retention time and peak width of target alkaliods. The retention time of various alkaloids were slightly reduced upon addition of ammonium acetate. The retention time of the analytes were reduced when the concentration of ammonium acetate was increased up to 25 mM. Further increase in the ammonium acetate concentration did not have significant effect on the retention time. The effect of the concentration of ammonium acetate on the width of the chromatographic peaks was somewhat different. Low concentration ammonium acetate has no observable effect on the peak width. At higher concentration, a substantial increase in the peak width was observed.

Figure 3.5 shows the effect of ammonium acetate on APCI and ESI ion intensities. For APCI, the signal intensity was found to maximize at 50 mM of ammonium acetate. For

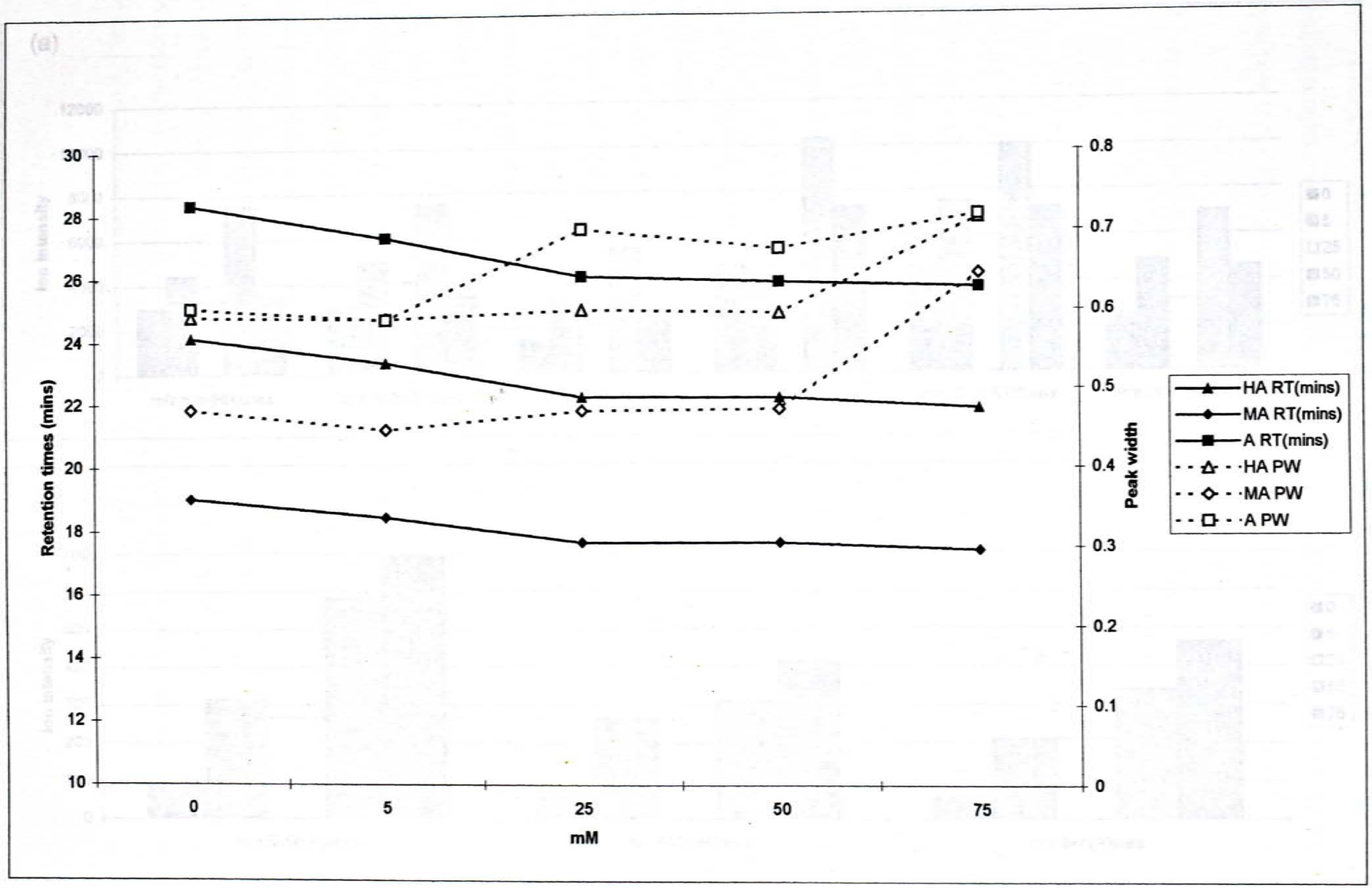


Figure 3.4 Effects of ammonium acetate on retention times and peak width of aconitine-type alkaloids with 0.5 % TEA and 1 % acetic acid

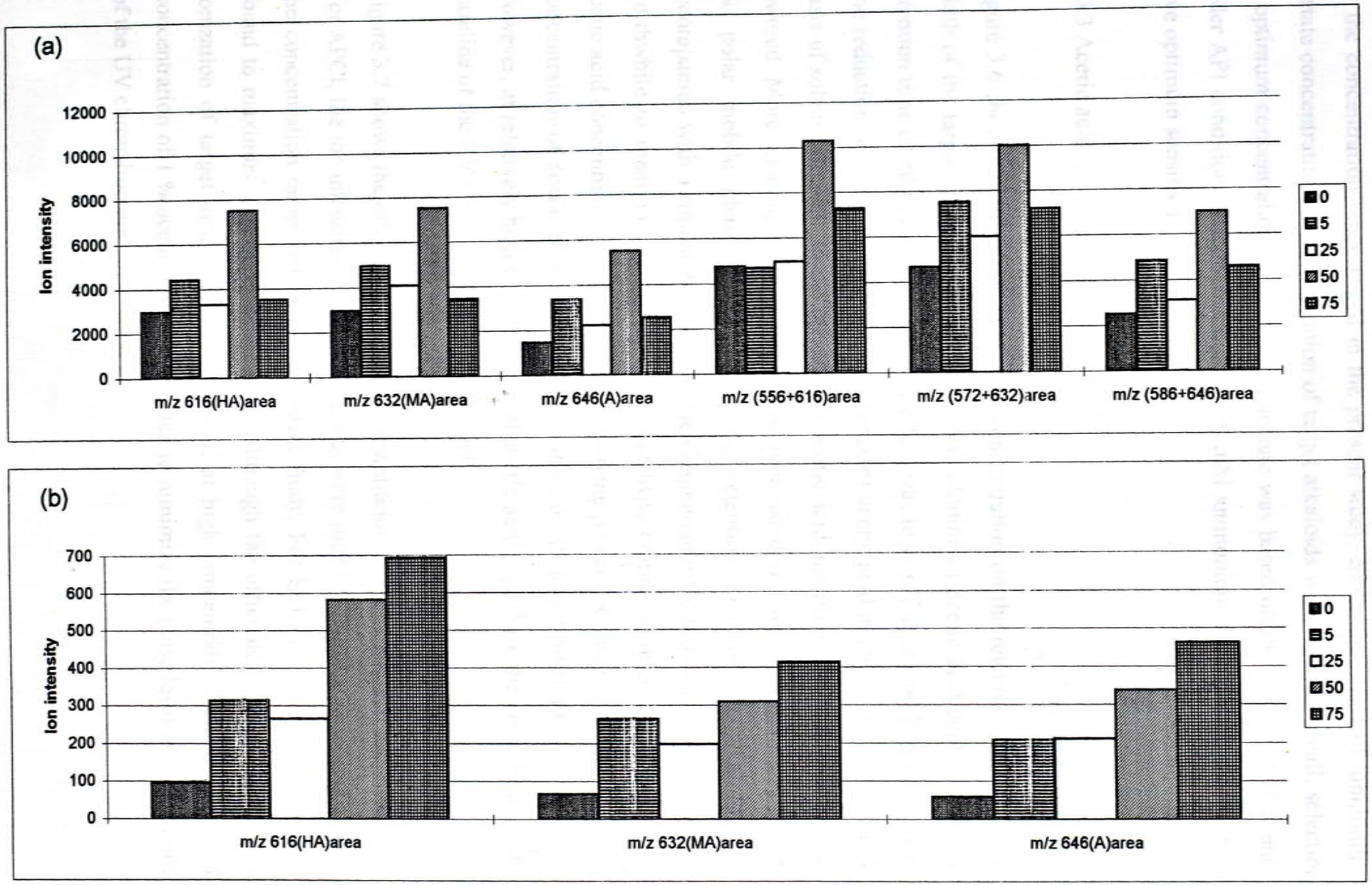


Figure 3.5 Effects of ammonium acetate concentration on: (a) APCI ion intensity (b) ESI ion intensity of aconitine-type alkaloids with 0.5 % TEA and 1 % acetic acid

ESI, the ion intensities of aconitine-type alkaloids were found to increase proportionally for the concentration range tested in the present study. Since the effect of ammonium acetate concentration on the separation of target alkaloids was relatively small, selection of optimum concentration of ammonium acetate was based on the ionization efficiency under API conditions. A concentration of 50 mM ammonium acetate was selected as it gave optimum sensitivity in APCI method.

3.3.3 Acetic acid

Figure 3.6 shows the effect of acetic acid concentration on the retention time and peak width of the target alkaloids. It was found that addition of acetic acid could reduce the retention time of all target alkaloids. The effect was level off at around 2 % acetic acid. The reduction of retention time in the presence of acetic acid might be explained on the basis of solvent strength. In the presence of acetic acid, the pH of the mobile phase was lowered. More alkaloid molecules would be protonated and would spend more time in the polar mobile phase. Reduction of the retention time of the alkaloids was accompanied with a similar reduction of the chromatographic peak width. It is probably worthwhile to mention that mesaconitine and hypaconitine were not resolved at low acetic acid concentration (< 0.2 %). The resolving power (R) was only 1.6. At higher concentration of acetic acid, complete separation of all target alkaloids was obtained. However, at relatively high concentration of acetic acid (≥ 2 %), the noise level of the baseline of the UV chromatogram was very high.

Figure 3.7 shows the effect of acetic acid concentration on APCI and ESI ion intensities. For APCI, the ion intensities of target alkaloids were found to increase proportionally for the concentration range tested in the present study. For ESI, the signal intensity was found to maximize at 2 % of acetic acid. Although the efficiencies of separation and ionization of target alkaloids were enhanced at high concentration of acetic acid, a concentration of 1 % acetic acid was selected to minimize the noise level of the baseline of the UV chromatogram.

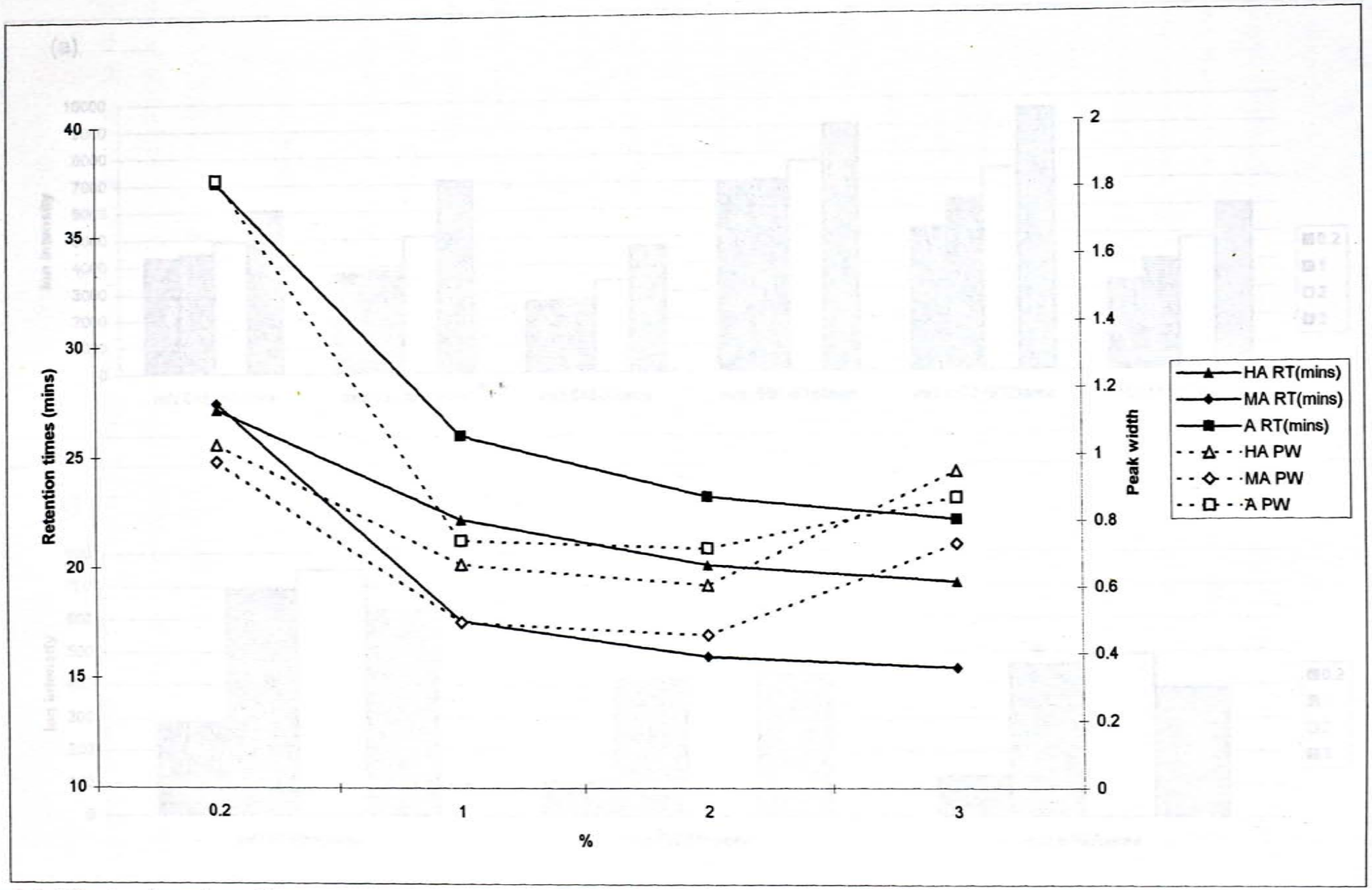


Figure 3.6 Effects of acetic acid on retention times and peak width of aconitine-type alkaloids with 0.5 % TEA and 50 mM ammonium acetate

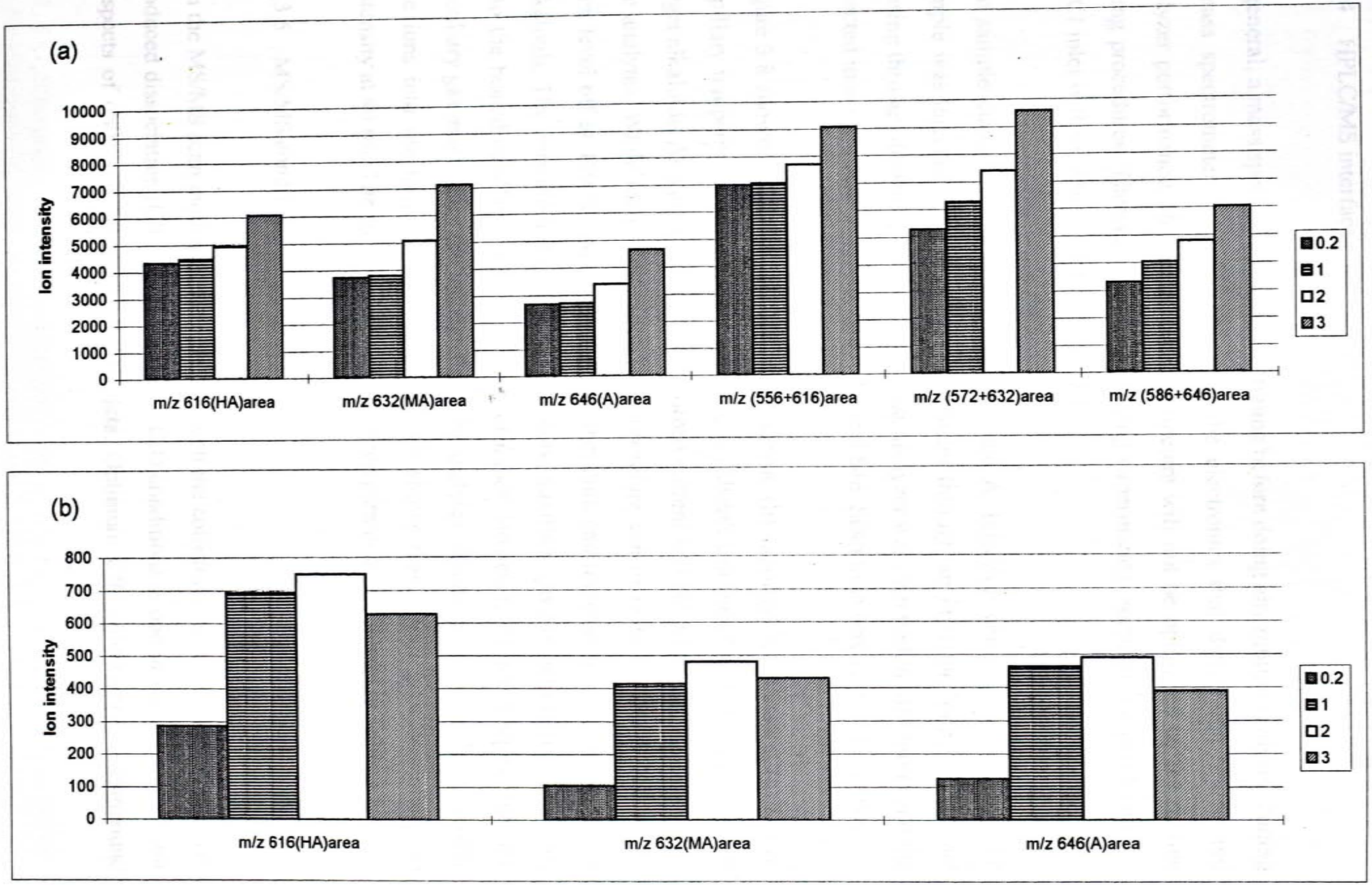


Figure 3.7 Effects of acetic acid concentration on: (a) APCI ion intensity and (b) ESI ion intensity of aconitine-type alkaloids with 0.5 % TEA and 50 mM ammonium acetate

3.3.4 HPLC/MS interface

In general, a mass spectrometer requires tuning before doing any routine analysis. Tuning a mass spectrometer involves optimizing the electronics that dictate ion source and analyzer performance. However, some parameters will not be optimized by the routine tuning procedures. Therefore, in the following experiments, some of the parameters in APCI inlet will be optimized. See Table 3.1.

The sample consisted of 40 ppm MA, HA, and A. Injection volume was 0.5 μL. The sample was injected into the mass spectrometer through an injection loop, i.e. without passing through the HPLC column. The mass analyzer was operated in SIM mode and the selected ions were 556, 572, 586, 616, 632, and 646. Scan time was set at 0.5 seconds.

Figure 3.8 showns the effect of (a) corona current, (b) vaporizer temperature, (c) heated capillary temperature, (d) auxiliary gas, and (e) sheath gas pressure on the ion yield of target alkaloids. As shown in Figure 3.8, corona current will not affect the ion intensity of the analytes. While increasing vaporizer temperature can increase the ion signal. It will then level off at 450 °C. Also, capillary temperature can increase the ion signals of the alkaloids. The maximum is at 200 °C. In theory, auxiliary gas can help to focus the ions into the heated capillary, and dry the API chamber. However, the result shows that the auxiliary gas may reduce the ion signals of the analytes. Sheath gas can also help to focus the ions into the heated capillary. The result shows that it will give maximum ion intensity at 40 psi. The summary of the optimized parameters is shown in Table 3.2.

3.3.5 MS/MS conditions

In the MS/MS scan modes, as ions pass through the collision cell they undergo collision-induced dissociation (CID). Optimizing the CID conditions is one of the most important aspects of obtaining optimum MS/MS data. Optimum CID conditions are compound

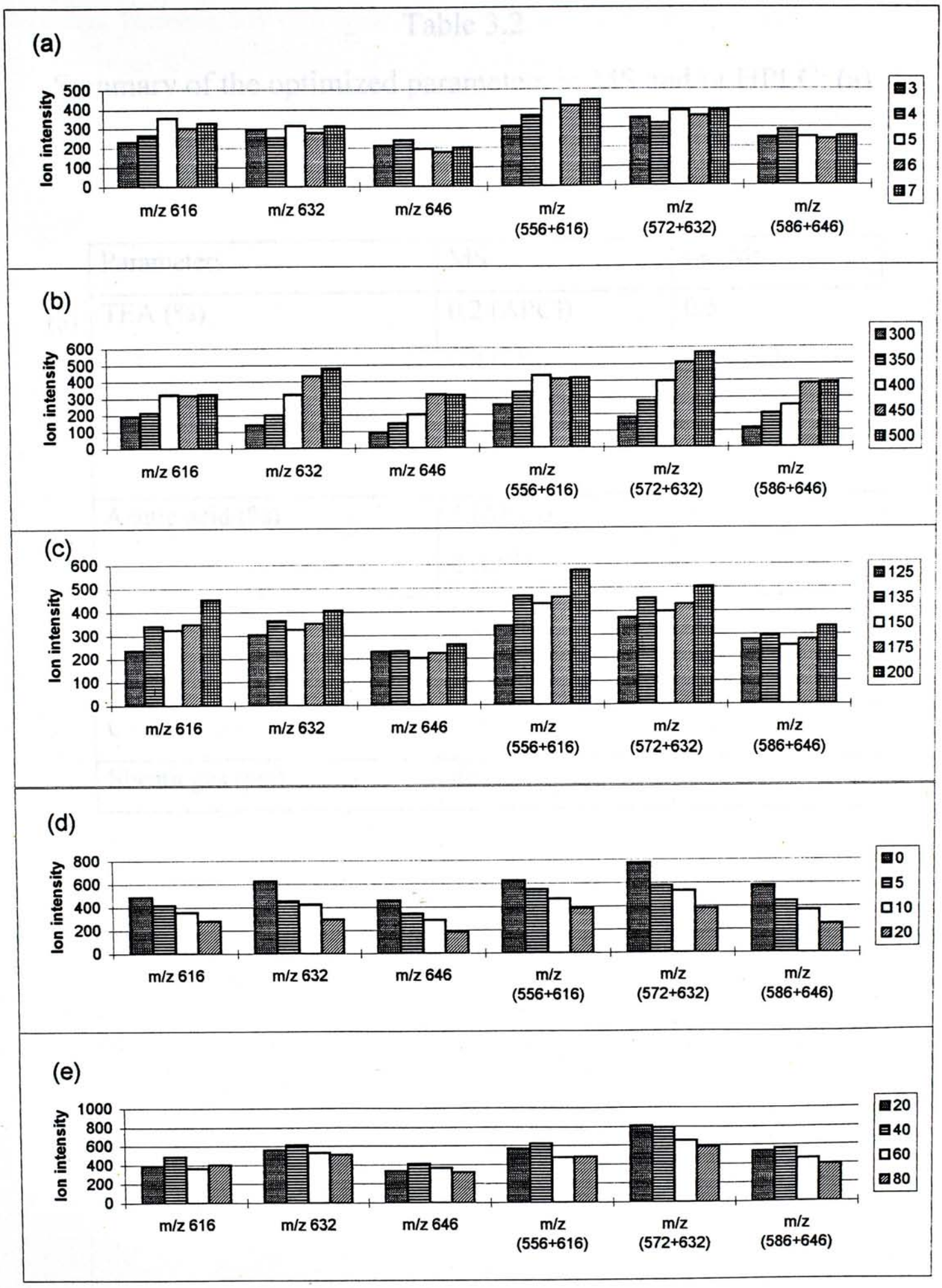


Figure 3. 8 Optimization of APCI interface: (a) corona current (b) vaporizer temperature (c) capillary temperature (d) auxiliary gas (e) sheath gas

Table 3.2

Summary of the optimized parameters in MS and/or HPLC: (a) mobile phase composition, and (b) API inlet

	Parameters	MS	LC/MS
(a)	TEA (%)	0.2 (APCI) 0.2 (ESI)	0.5
	Ammonium acetate (mM)	50 (APCI) 75 (ESI)	50
	Acetic acid (%)	3 (APCI) 2 (ESI)	1
(b)	Corona Current (μA)	5	
	Vaporizer temperature (°C)	450	
	Capillary temperature (°C)	200	
	Sheath gas (psi)	40	

dependent. Therefore, they must be set for each compound or a class of compounds. The CID conditions to be optimized are as follows: collision gas pressure, collision cell offset voltage (collision energy), and MS/MS correction factor (MSMSC).The collision gas pressure and collision energy determine the extent of fragmentation. While MSMSC helps to determine the intensity and peak shape of the daughter ion peaks.

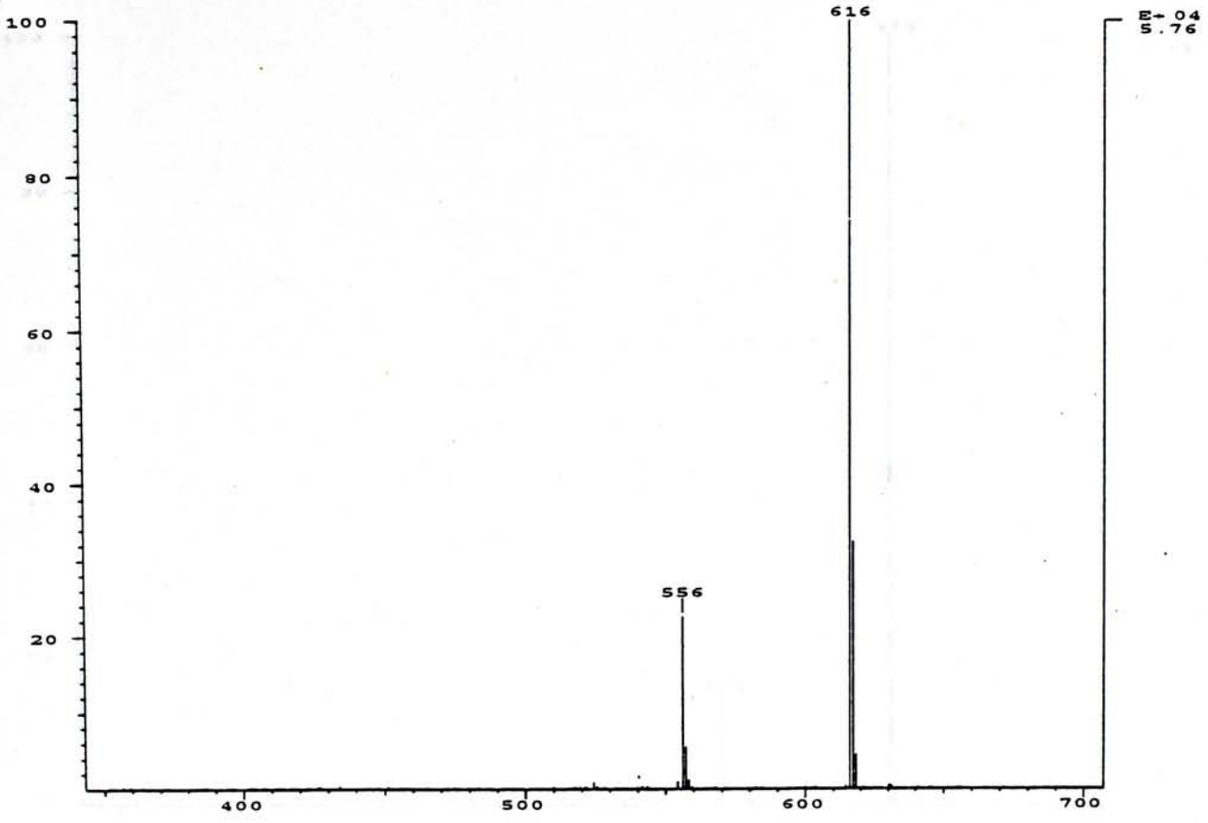
The samples composed of 200 ppm of HA, MA, and A. Injection volume was 0.2 μL. The sample was injected into the mass spectrometer without passing through the HPLC column. In the present experiment, APCI method was used. The mass analyzer was operated in daughter scan mode. The selected ions in Q1MS were m/z 616, 632, and 646. While Q3MS scanned from 50 to 700 amu in 0.5 seconds.

When comparing MS and MS/MS mass spectra, see Figure 3.9, 3.10, and 3.11, besides the parent ion $[M+H]^+$ (m/z 616 for HA, m/z 632 for MA, and m/z 646 for A), MS mass spectrum would give only one fragment ion $[M+H-CH_3COOH]^+$ (m/z 556 for HA, mz/ 572 for MA, and m/z 586 for A). However, MS/MS mass spectrum would give more fragments ions. These fragmentation pattern can serve as a valuable tool to confirm the identities of the aconitine-type alkaloids. In addition, when MS/MS conditions were set at 2.4 mTorr of collision gas pressure, -35 V of collision energy, and 0 % of MSMSC, besides the most intense fragment ion (m/z 556 for HA, m/z 572 for MA, and m/z 586 for A), other fragment ions can also be observed.

3.4 Conclusions

It has been shown that by increasing the concentration of TEA, ammonium acetate, and acetic acid, the t_R of the analytes will be shortened. In addition, the amount of acetic acid can enhance ion intensity of the analytes. If the mobile phase composition consisted of 77 % buffer, 13 % ACN, and 10 % THF, where the buffer containing 0.5 % TEA, 50 mM ammonium acetate, and 1 % acetic acid, then the aconitine-type alkaloids can be

(a)



(b)

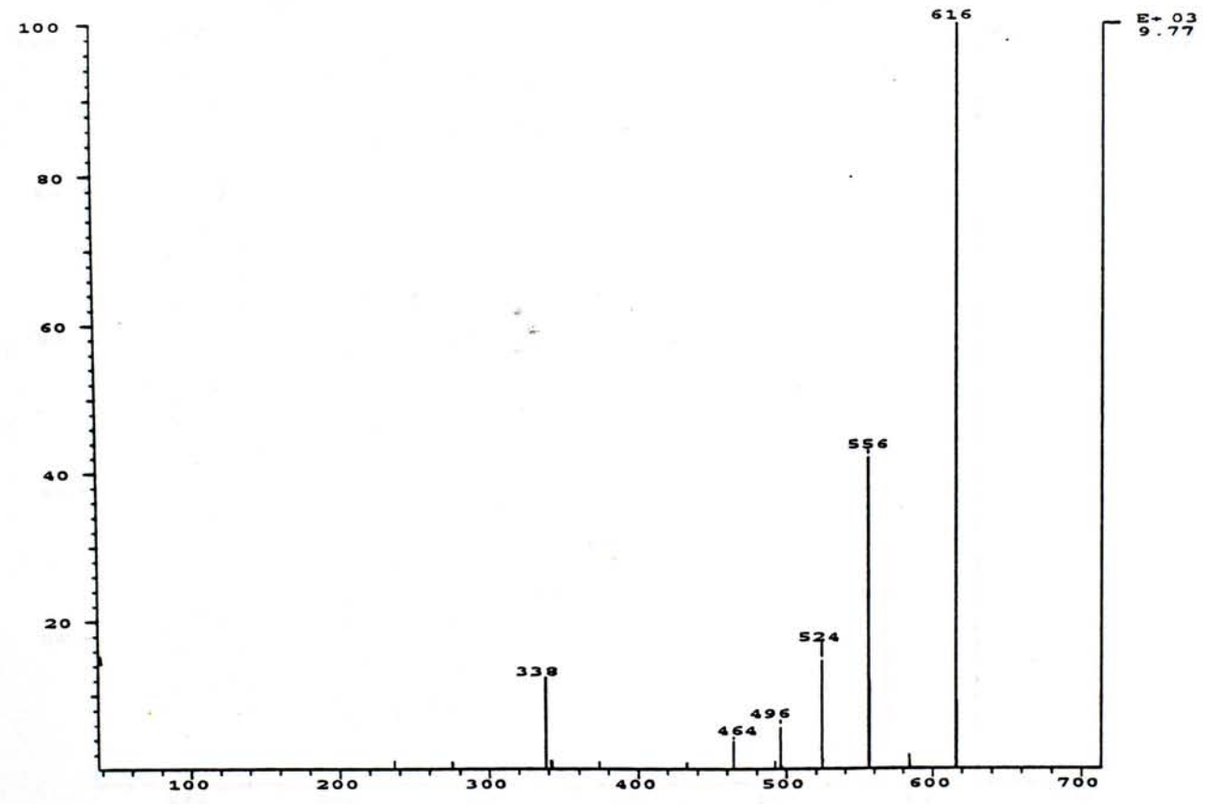
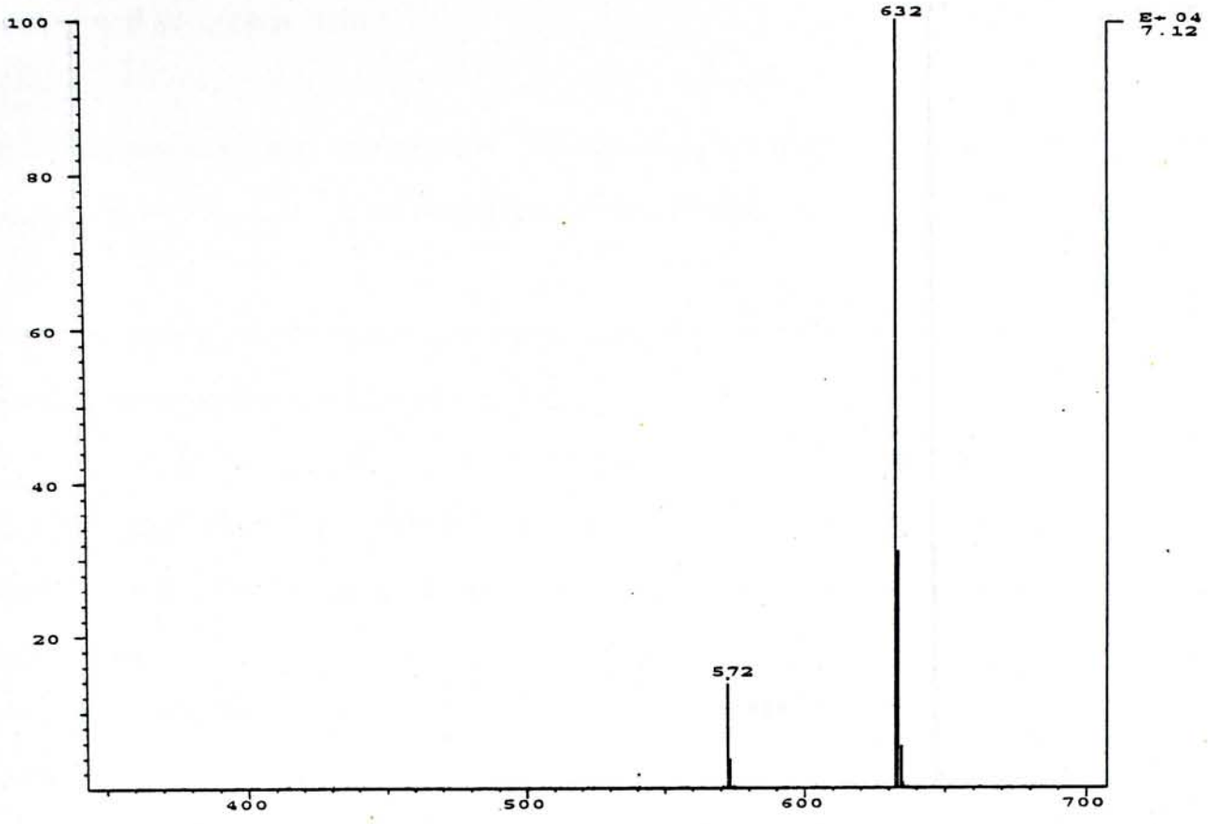


Figure 3.9 (a) MS spectrum of HA (b) MS/MS spectrum of HA (2.4 mTorr, -35 V, 0 % MSMSC)

(a)



(b)

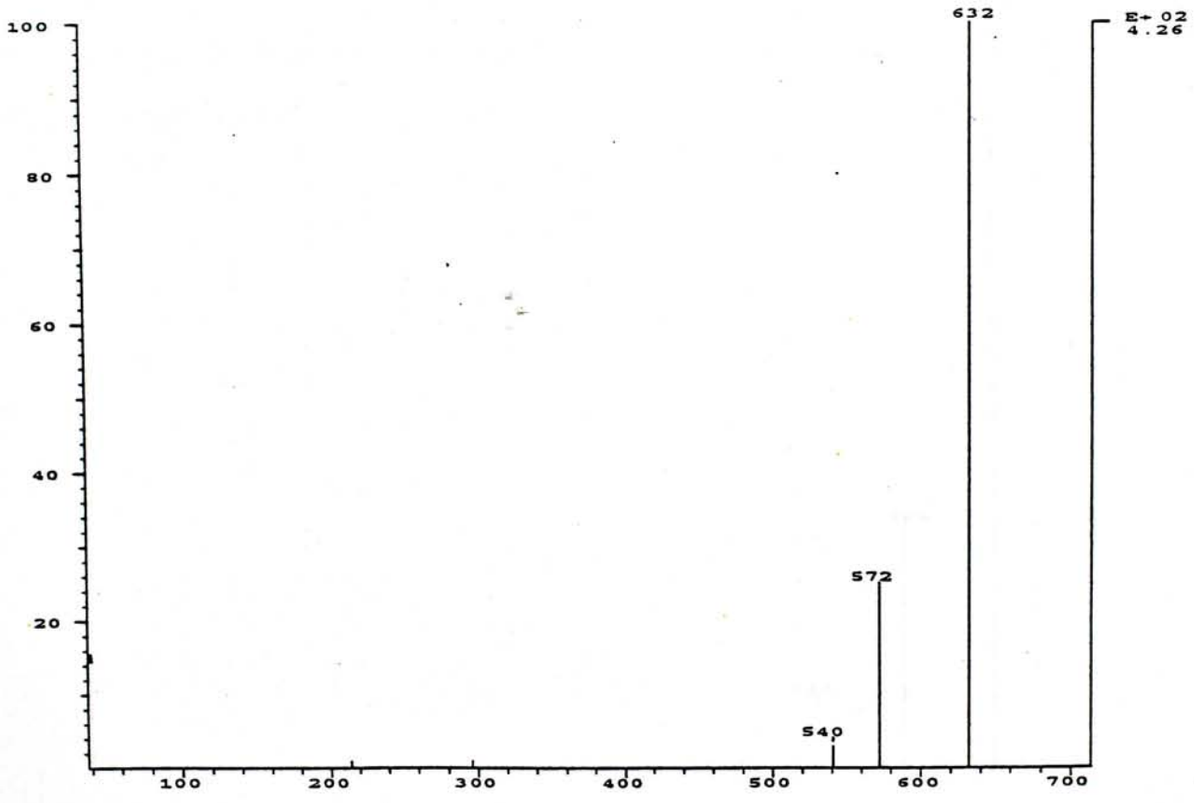


Figure 3.10 (a) MS spectrum of MA (b) MS/MS spectrum of MA (2.4 mTorr, -35 V, 0 % MSMSC)

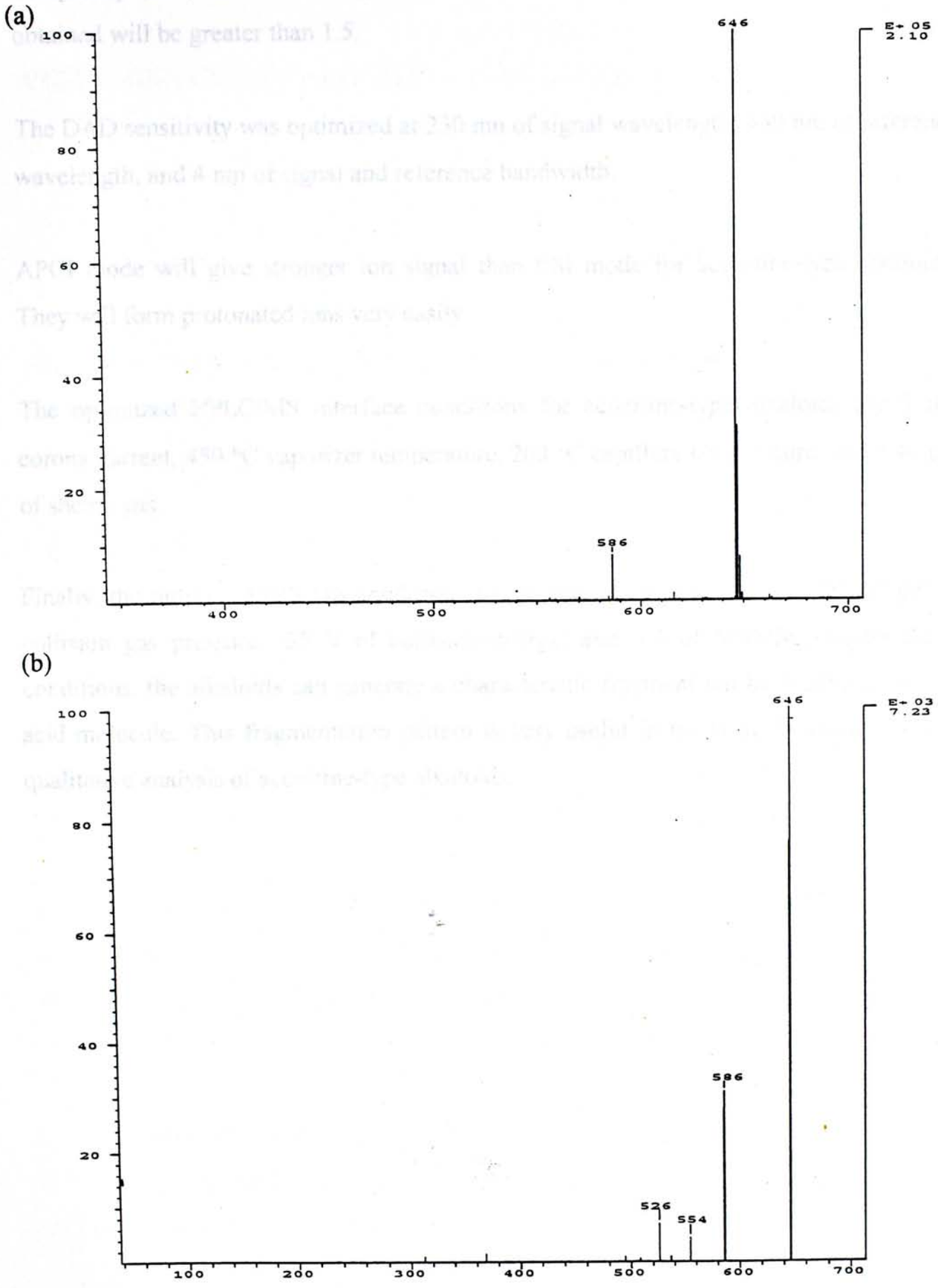


Figure 3.11 (a) MS spectrum of A (b) MS/MS spectrum of A (2.4 mTorr, -35 V, 0 % MSMSC)

completely resolved within 30 minutes of chromatographic run. All the values of R obtained will be greater than 1.5.

The DAD sensitivity was optimized at 230 nm of signal wavelength, 450 nm of reference wavelength, and 4 nm of signal and reference bandwidth.

APCI mode will give stronger ion signal than ESI mode for aconitine-type alkaloids. They will form protonated ions very easily.

The optimized HPLC/MS interface conditions for aconitine-type alkaloids are 5 μA corona current, 450 °C vaporizer temperature, 200 °C capillary temperature, and a 40 psi of sheath gas.

Finally, the optimized MS/MS conditions for aconitine-type alkaloids are 2.4 mTorr of collision gas pressure, -35 V of collision energy, and 0% of MSMSC. Under these conditions, the alkaloids can generate a characteristic fragment ion by lossing an acetic acid molecule. This fragmentation pattern is very useful in terms of selectivity for the qualitative analysis of aconitine-type alkaloids.

CHAPTER FOUR

DETERMINATION OF ACONITINE-TYPE ALKALOIDS IN ACONITE ROOTS

4.1 INTRODUCTION

In quantitative analysis of HPLC, higher precision is obtained by using internal standard because the uncertainties introduced by sample injection, flow rate, and variations in column conditions are minimized. Moreover, internal standard in MS can improve precision by reducing the uncertainties due to sample introduction, gas flow, and variations in ionization conditions.

One example of standard addition method is to split the unknown sample solution into five portions. Different amount of standard analytes would be added into four portions of the samples separately. All the samples are diluted to the same volume. The analytical responses can be plotted to generate a linear correlation graph. The amount of the analytes in the sample can be found frm extrapolate back to zero addition of standard.

4.2 EXPERIMENTAL

Search for internal standard: Over a hundred of organic molecules have been tested as internal standards for the HPLC/MS method of aconitine-type alkaloids. Each compound was dissloved with 100 μL ACN. Then 5 μL of the sample was injected into HPLC/APIMS system.

Evaluation of precision of measurement: A sample consisted of 6 ppm HA, MA, A, and 50 ppm DAB was used to evaluate the precision of UV and MS detectors. The sample was injected into HPLC/APIMS system for seven times.

Evaluation of external calibration mehtod: Calibration curves (0.06 ppm, 0.6 ppm, 1.5 ppm, 6 ppm, 60 ppm, 150 ppm, 300 ppm, 600 ppm, 1500 ppm) were prepared using standard solutions of each alkaloid. The internal standard was added to each solution, 5 μL aliquots of the solutions were injected into the HPLC/APIMS system.

Evaluation of standard addition method: 50 μL of 30 ppm HA, MA, and A were separately pipetted into five different vials. Then 10 μL, 20 μL, 30 μL, and 40 μL of 60 ppm HA, MA, and A were separately added into four vials, respectively. Finally, different volumes of ACN were added into the five vials such that each vial would contain 200 μL solution. The injection volume was 5μL.

Evaluation of limits of detection and quantitation: The conditions used in MS and MS/MS were 500 °C vaporizer temperature, 200 °C capillary temperature, 5 μA corona current, and 40 psi sheath gas. The sample was directly injected into the APCI source without passing through the HPLC column. On the other hand, the conditions used in HPLC/APIMS and HPLC/APIMS/MS were 450 °C vaporizer temperature, 135 °C capillary temperature, 5 μA corona current, and 40 psi sheath gas. The sample was injected into the HPLC system.

Determination of aconitine-type alkaloids in aconite roots: Recovery rates of extracted alkaloids from root samples were determined by addition of HA, MA, and A at 187.5 μg to the root samples. The recoveries of alkaloids were compared with those from the root samples, to which no alkaloids had been added.

4.3 RESULTS AND DISCUSSION

4.3.1 Selection of internal standard

There are several selection criterias for an internal standard [49]. The internal standard's t_R should be close to the aconitine-type alkaloids' t_R but not too close that it cannot be resolved from the analytes. The UV absorption of internal standard should be similar to aconitine-type alkaloids. In addition, it should produce ion signals as the analytes do.

After trying for nearly one hundred compounds (see Appendix), only DAB and PSO are found to fulfill most of the criterias for the internal standard of aconitine-type alkaloids (see Figure 4.1 and 4.2). The figures show that DAB and PSO can resolve completely from the alkaloids, their UV signals are comparable to these alkaloids. Nevertheless, from the MS chromatogram, it shows that PSO has much weaker signal than aconitine-type alkaloids. Therefore, only DAB will be studied more deeply in the following section in order to evaluate how good it can be used as the internal standard of aconitine-type alkaloids.

4.3.2 Method validation

4.3.2.1 Precision of measurement

Precision of a method is the degree of closeness of the results and is reported as a percentage of relative standard deviation (% RSD).

4.3.2.2 Accuracy of measurement

Linearity

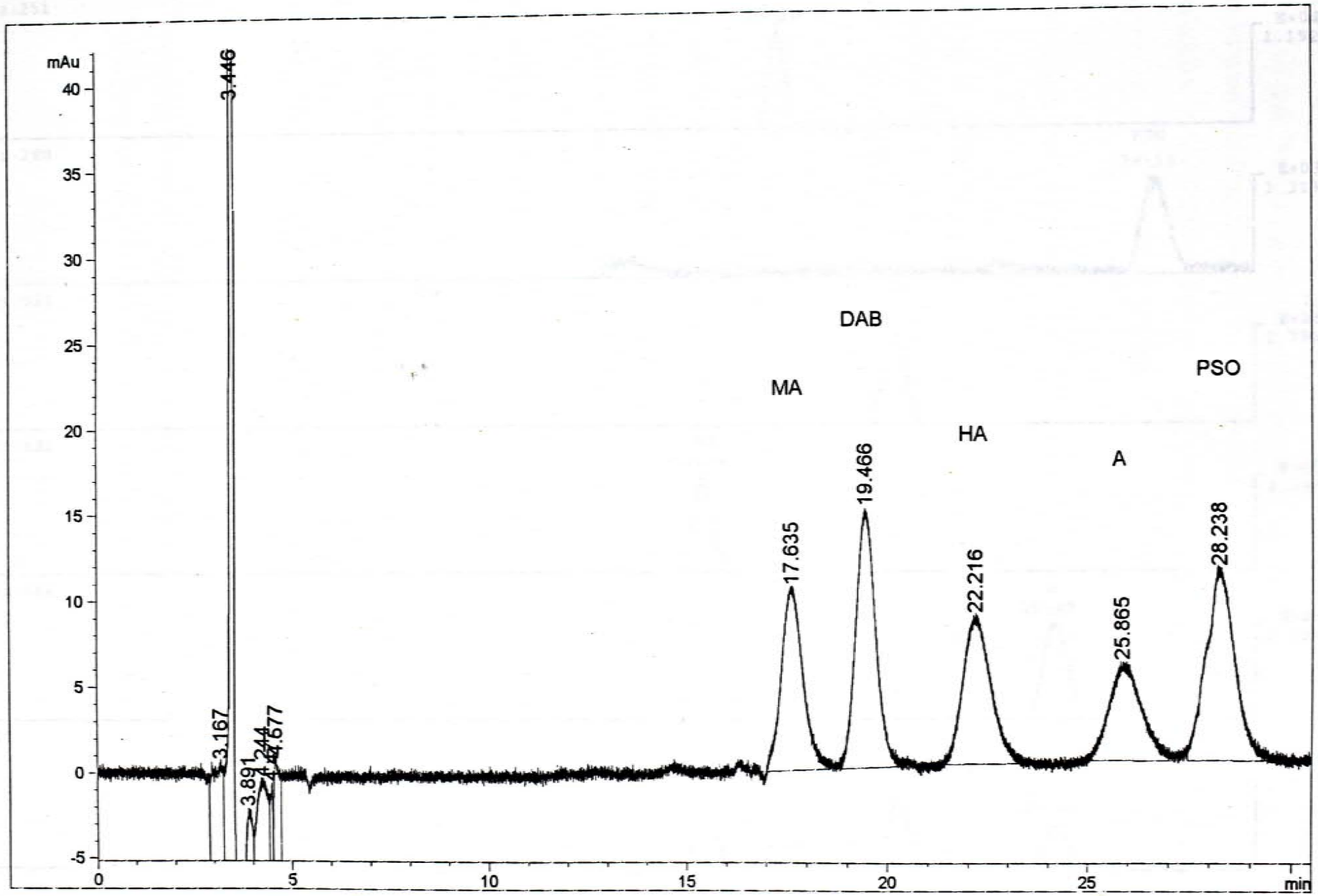


Figure 4.1 HPLC chromatogram of 120 ppm HA, MA, and A, 50 ppm DAB, and 20 ppm PSO. Column: Microsorb C_{18} (25cm X 4.6mm I.D.) Eluents: Buffer of pH 4(0.5 % TEA, 50 mM ammonium acetate, 1 % acetic acid)-ACN-THF (77:13:10) Flow rate: 0.8 mL/min. Detection: UV signal wavelength 230 nm, reference wavelength 450 nm, both signal and reference bandwidth are 4 nm.

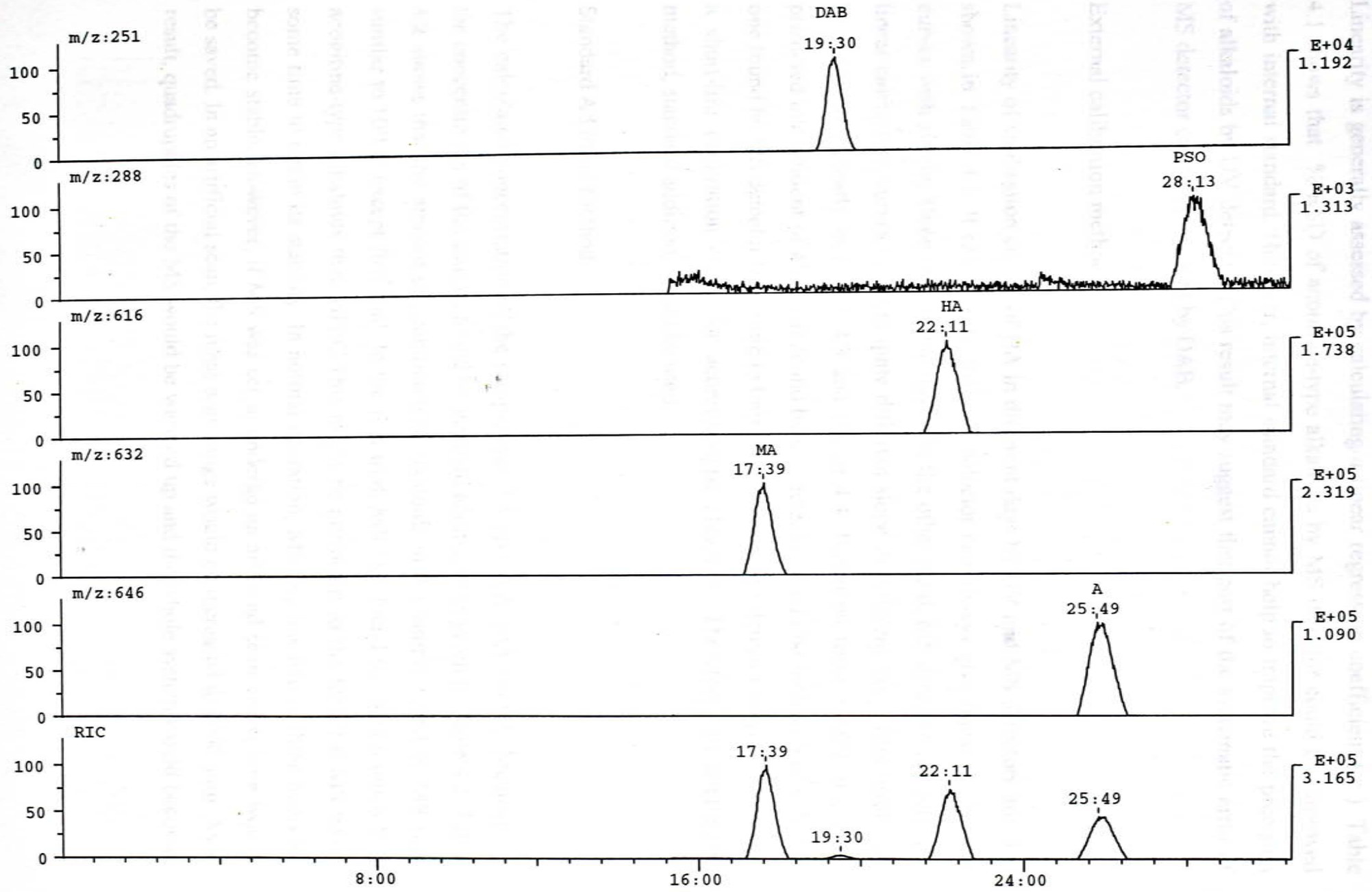


Figure 4.2 MS chromatogram of aconitine-type alkaloids (HA: m/z 616 $[M+H]^+$, MA: m/z 632 $[M+H]^+$, A: m/z 646 $[M+H]^+$) and internal standards (DAB: m/z 251 $[M+H+TEA]^+$, PSO: m/z 288 $[M+H+TEA]^+$) MS conditions: Vaporizer 400 °C, heated capillary 135 °C, sheath gas 40 psi, auxiliary gas 5 units, corona 5 μA, SIM scan type, 1 second of scan time, 1 u of scan width, electron multiplier 1100 V.

Linearity is generally assessed by calculating a linear regression coefficient (r). Table 4.1 shows that % RSD of aconitine-type alkaloids by MS detector could be improved with internal standard. However, internal standard cannot help to improve the precision of alkaloids by UV detector. This result may suggest that part of the systematic error of MS detector can be eliminated by DAB.

External calibration method

Linearity of calibration curves of HA in different days by UV and MS detectors are also shown in Table 4.1. It can be seen that UV detector can always give linear calibration curves with similar slope in different days. On the other hand, MS detector can generate linear calibration curves but with quite different slope in different days. This result can be seen more clearly in Figure 4.3 and Figure 4.4. Based on these results, it can be predicted that amount of alkaloids found by UV detector would be inconsistent with the one found by MS detector from time to time. It is because MS detector may only generate a short-life calibration curve for aconitine-type alkaloids. Therefore, an alternative method, standard addition, would be used.

Standard Addition Method

The calculated concentration of the sample was 7.5 ppm HA, MA, and A. Meanwhile, the concentration of the sample found by standard addition was given in Table 4.2. Table 4.2 shows that the amount of aconitine-type alkaloids in the sample found by MS was similar to HPLC except first trial. In the first trial, MS was found to contain much less aconitine-type alkaloids than HPLC.This might be attributed to the fact that MS takes some time to retain its stability. In normal operation, MS requires two to three hours to become stable. However, if MS was set to undergo an artificial scan, much time would be saved. In an artificial scan, the mass scan range would be increased to 2000 amu. As a result, quadrupoles of the MS would be warmed up and the whole system would become

Table 4.1

Precision of aconitine-type alkaloids and linearity of calibration curves of HA in different days by UV and MS detectors

Peak Area	Precision by UV detector (%RSD, n=7)	Precision by MS detector (%RSD, n=7)
HA	6.35	8.49
MA	4.02	7.05
A	11.3	10.8
HA/DAB	6.62	5.85
MA/DAB	3.95	6.32
A/DAB	11.3	7.99
	Linearity by UV detector	Linearity by MS detector
HA-Day1	y=0.009x + 0.0136 r=0.9999	y=0.1306x - 0.1124 r=0.9995
HA-Day2	y=0.0089x + 0.0314 r=0.9998	y=0.1486x - 0.1707 r=0.9996
HA-Day3a	y=0.0091x + 0.0043 r=1.000	y=0.1705x - 0.0126 r=0.9999
HA-Day3b	y=0.0092x - 0.0283 r=0.9999	y=0.2162x - 0.3913 r=0.9991

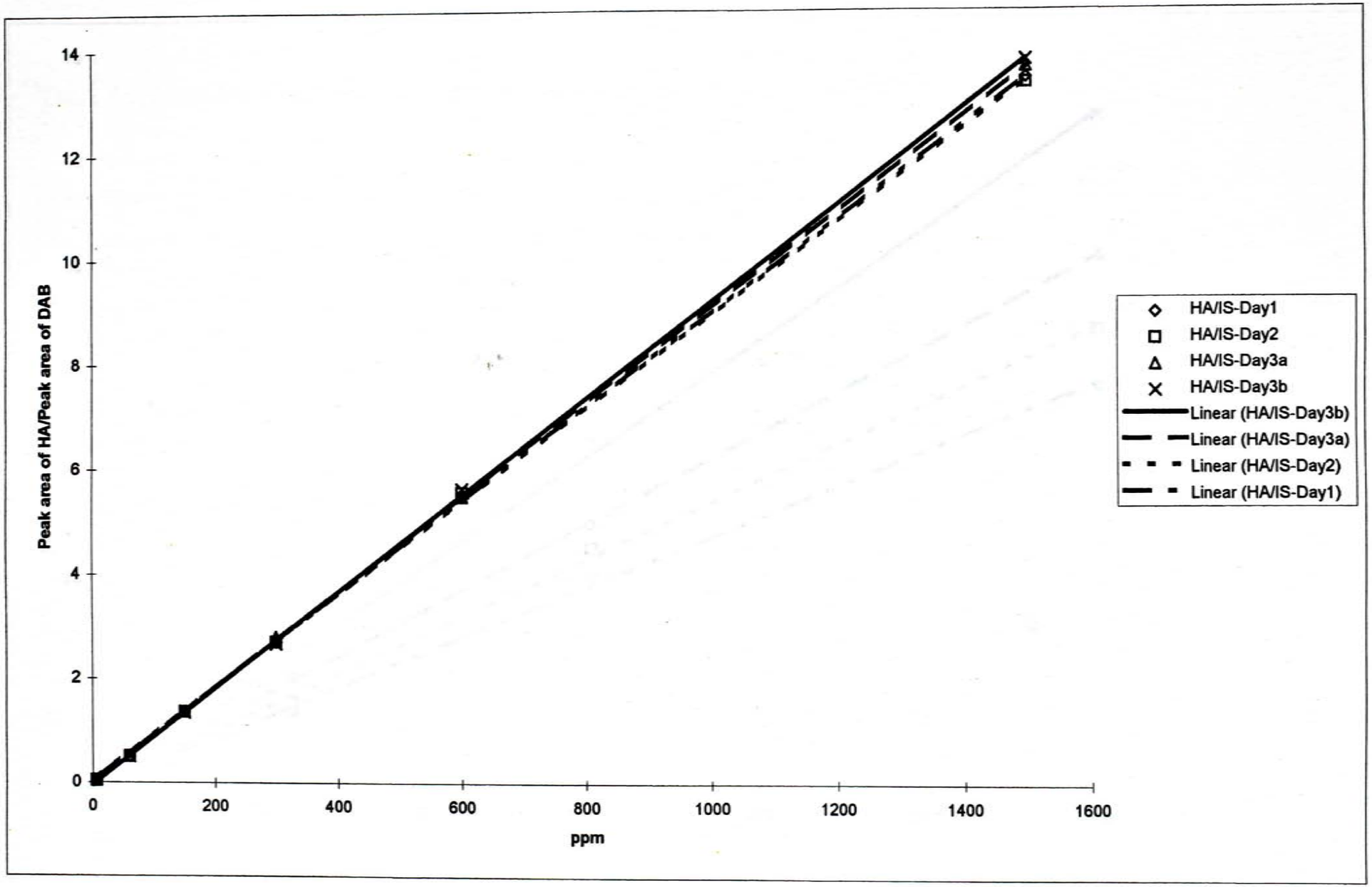


Figure 4.3 Calibration curves of HA by UV detector in different days. Chromatographic conditions as in Figure 4.1.

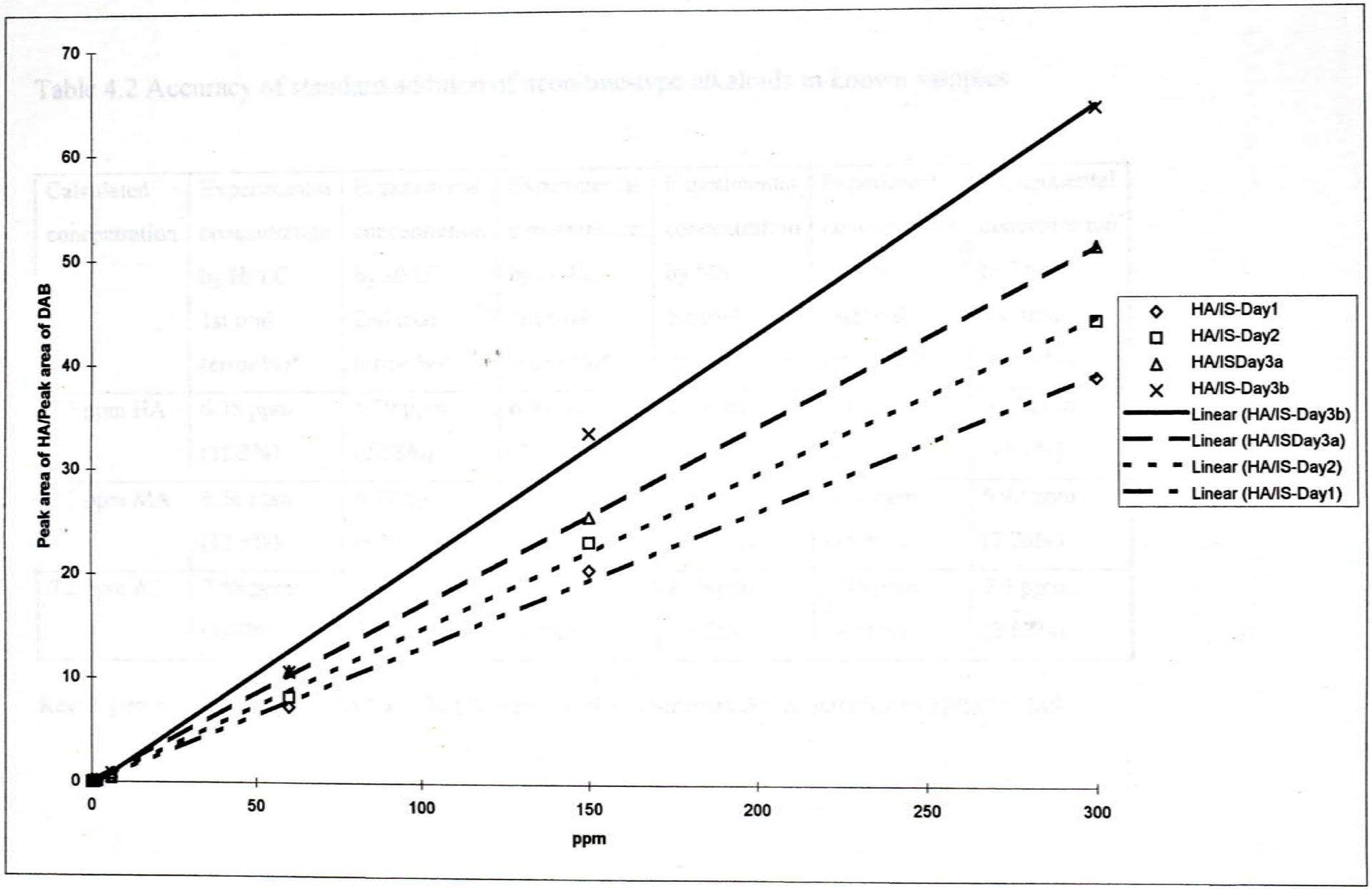


Figure 4.4 Calibration curves of HA by MS detector in different days. MS conditions as in Figure 4.2.

Table 4.2 Accuracy of standard addition of aconitine-type alkaloids in known samples

Calculated concentration	Experimental concentration by HPLC 1st trial (error %)*	Experimental concentration by HPLC 2nd trial (error %)*	Experimental concentration by HPLC 3rd trial (error %)*	Experimental concentration by MS 1st trial (error %)*	Experimental concentration by MS 2nd trial (error %)*	Experimental concentration by MS 3rd trial (error %)*
7.5 ppm HA	6.35 ppm (15.3%)	5.79 ppm (22.8%)	6.97 ppm (7.07%)	4.2 ppm (44%)	5.95 ppm (20.7%)	6.52 ppm (13.1%)
7.5 ppm MA	6.56 ppm (12.5%)	6.77 ppm (9.73%)	6.58 ppm (12.3%)	4.91 ppm (34.5 %)	6.34 ppm (15.5%)	6.92 ppm (7.73%)
7.5 ppm A	7.58 ppm (1.07%)	7.31 ppm (2.53%)	8.25 ppm (10%)	4.78 ppm (36.3%)	6.56 ppm (12.5%)	7.3 ppm (2.67%)

Key: * { error % = | X - 7.5 | / 7.5 * 100 % } Where X is the experimental concentration by HPLC or MS

stable more quickly. From experimental observation, if a parameter of MS, RF heatsink temperature, reach 37 °C, the system would be stable.

Table 4.2 also suggests that if the system is stable, accuracy of the standard addition method would vary from 1.07 % to 22.8 %.

4.3.2.3 Limits of detection and quantitation

Limit of detection (LOD) is the amount of analyte which can be reliably detected under the stated experimental conditions. In general, it would be the concentration of a compound that will give rise to a signal-to-noise ratio of 3.

It has been found that LOD of aconitine-type alkaloids in HPLC is 7.5 ng, MS is 7.5 pg, MS/MS is 75 pg, HPLC/APIMS is 75 pg, and HPLC/APIMS/MS is 750 pg. Therefore, MS has much lower LOD than HPLC.

Limit of quantitation (LOQ) is the amount of analyte which can be reliably quantified under the stated experimental conditions. For the determination of a drug in biological fluid, the LOQ may even be taken as an analyte concentration giving a %RSD as high as 20.

It has been found that LOQ of aconitine-type alkaloids in HPLC is 30 ng and HPLC/APIMS is 300 pg. LOD and LOQ of aconitine-type alkaloids are shown in Table 4.3.

4.3.3 Determination of aconitine-type alkaloids in aconite roots

Recovery rates of extracted alkaloids from root samples were determined by addition of HA, MA, and A at 187.5 μg to the root samples. The recoveries of alkaloids were

Table 4.3

LOD and LOQ of aconitine-type alkaloids in different methods

Method	LOD	LOQ
HPLC	7.5 ng	30 ng
MS	7.5 pg	
MS/MS	75 pg	
HPLC/APIMS	75 pg	300 pg
HPLC/APIMS/MS	750 pg	

compared with those from the root samples, to which no alkaloids had been added. The recovery rates of aconitine-type alkaloids in raw Caowu are shown in Table 4.4. It can be seen that the recovery rates may vary from 80 % to 117 %.

In addition, HPLC chromatograms of the aconite roots are shown in Figure 4.5 and 4.6. Their MS chromatograms are shown in Figure 4.7 and 4.8. From Figure 4.5, it can be seen that only HA of cured Chuanwu can be identified. However, Figure 4.7 shows that HA, MA, and A are present in cured Chuanwu. These results demonstrate that trace amount of aconitine-type alkaloids not detectable by HPLC may still be detected by MS. It is consistent to the result that MS has lower detection limit than HPLC for these alkaloids.

The amounts of HA, MA, and A in the aconite roots are summarized in Table 4.5. It can be seen that raw aconite roots contain more aconitine-type alkaloids than cured roots. This implies that the processing of raw aconite roots can significantly reduce the amount of aconitine-type alkaloids and thus reduce the toxicity of the drug.

4.4. Conclusions

It has been shown that DAB can be used as an internal standard which can give good precision and linearity of aconitine-type alkaloids in HPLC/APIMS. The detection limit of MS is much lower than HPLC for the alkaloids. LOD of the former is 75 pg while the latter is 30 ng. However, HPLC is still useful for qualitative and quantitative analysis of the major component of alkaloids in the aconite roots.

In conclusion, on-line HPLC/APIMS is a method with high selectivity and sensitivity which can simultaneously identify and quantify the aconitine-type alkaloids in aconite roots.

Table 4.4

Recovery rates of aconitine-type alkaloids in raw Caowu

Compounds	HPLC (μg/g, %RSD)*	MS (μg/g, %RSD)*
Raw Caowu HA	210.3(23.02%)	195.5(19.11%)
Raw Caowu MA	179.4(17.18%)	168.2(26.25%)
Raw Caowu A	78.13(23.26%)	49.75(27.07%)
Spiked raw Caowu HA+187.5μg	396.9(19.81%)	414.2(28.65%)
Spiked raw Caowu MA+187.5μg	347.5(8.67%)	350.9(21.12%)
Spiked raw Caowu A+187.5μg	227.3(15.11%)	208.8(26.04%)
Recovery rates HA	99.52%	116.6%
Recovery rates MA	89.65%	97.44%
Recovery rates A	79.56%	84.83%

Key: * n = 5

Figure 4.5 HPLC chromatograms of (a) raw Chuanwu and (b) cured Chuanwu. Chromatographic conditions same as in Figure 4.1.

Figure 4.6 HPLC chromatograms of (a) raw Caowu and (b) cured Caowu. Chromatographic conditions same as in Figure 4.1.

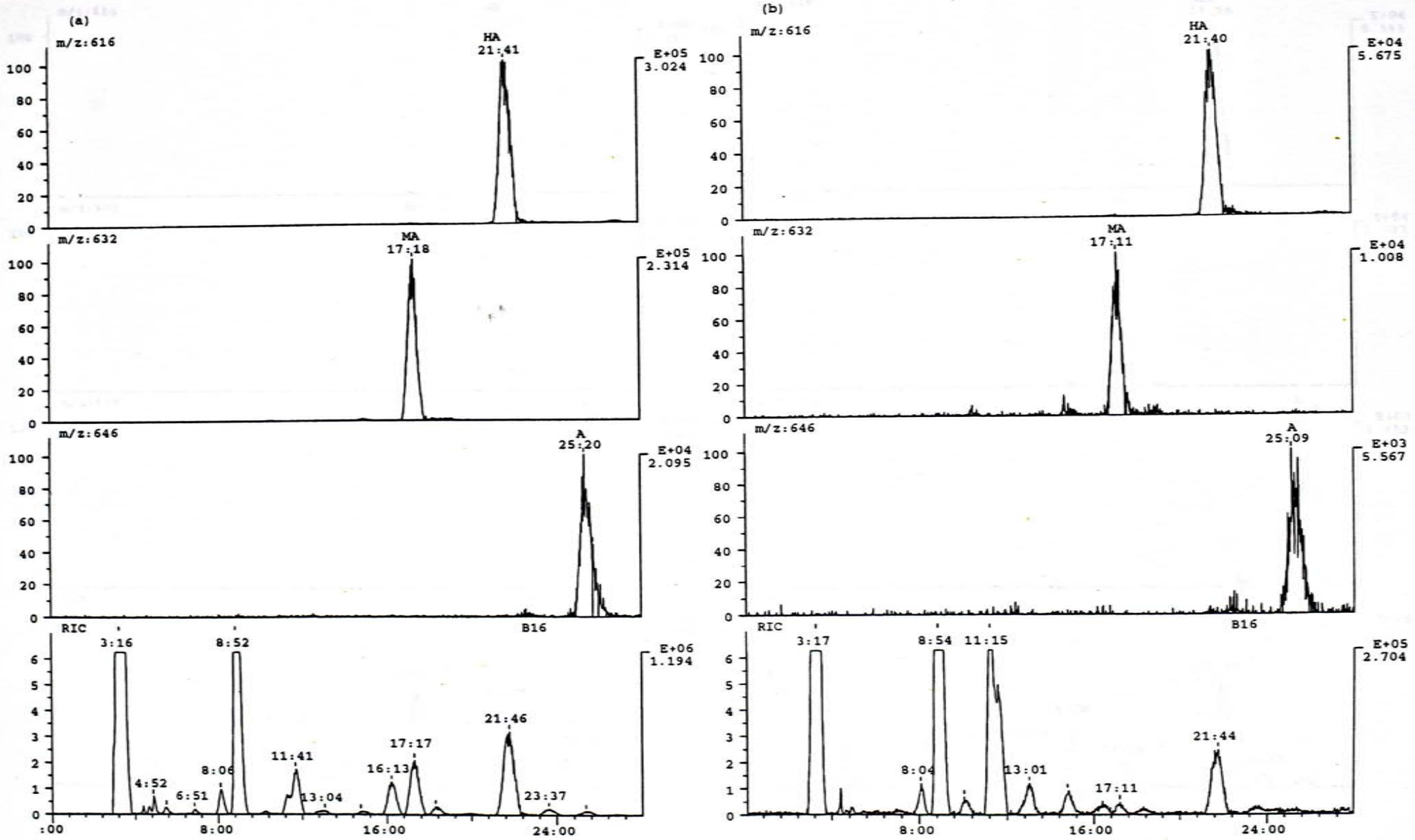


Figure 4.7 MS chromatograms of (a) raw Chuanwu and (b) cured Chuanwu. MS conditions: Vaporizer 500 °C, heated capillary 200 °C, sheath gas 40 psi, no auxiliary gas, corona 5 μA, full scan from 305 to 678 u, 1 second of scan time, electron multiplier 1100 V.

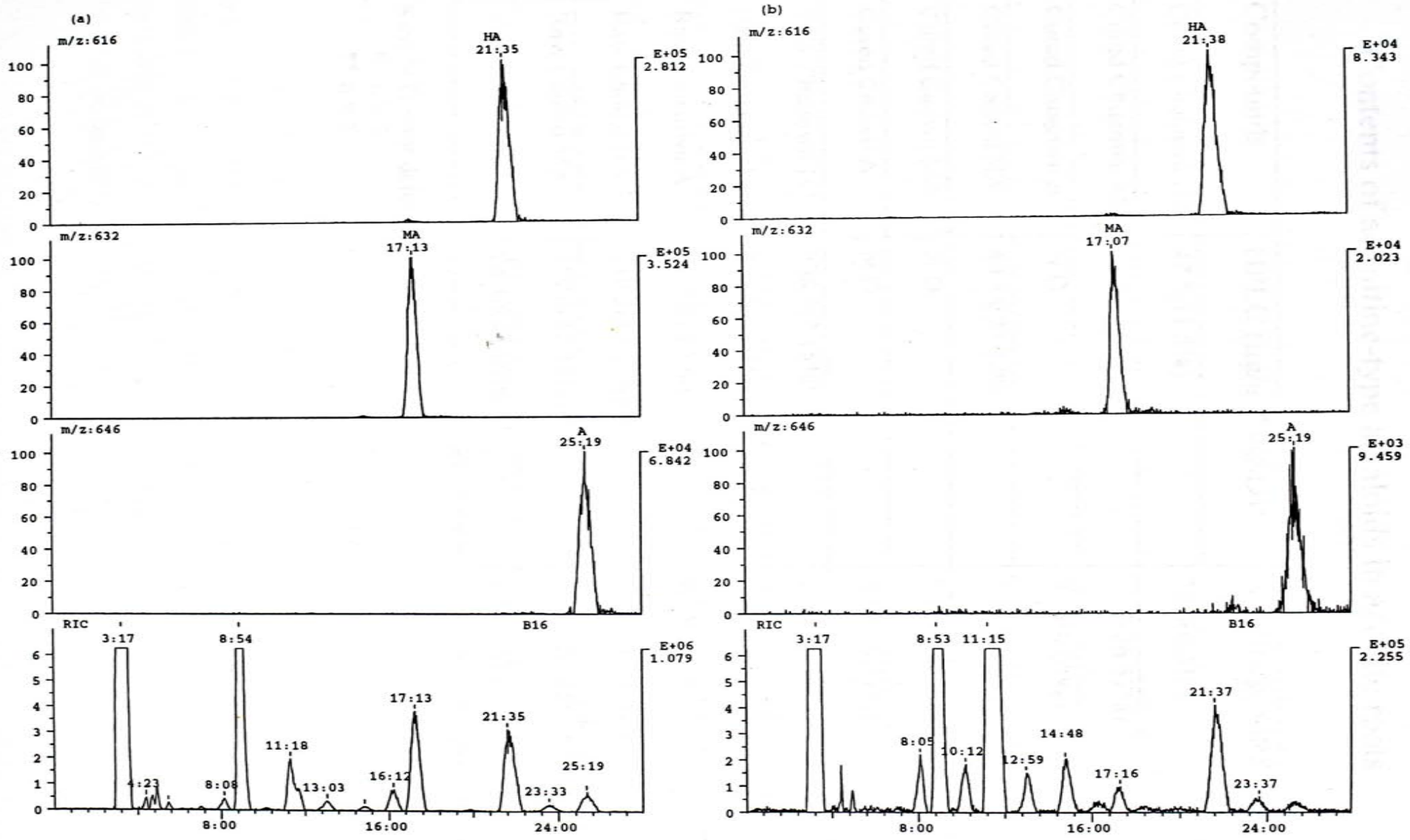


Figure 4.8 MS chromatograms of (a) raw Caowu and (b) cured Caowu. MS conditions same as in Figure 4.7.

Table 4.5

Contents of aconitine-type alkaloids in aconite roots

Compounds	HPLC (μg/g, %RSD)*	MS (μg/g, %RSD)*
Cured Chuanwu HA	47.5(11.5%)	30.6(6.51%)
Cured Chuanwu MA	N.D.	1.57(26.57%)
Cured Chuanwu A	N.D.	3.57(9.01%)
Cured Caowu HA	40.53(21.22%)	30.43(16.34%)
Cured Caowu MA	N.D.	5.1(11.93%)
Cured Caowu A	N.D.	3.87(10.77%)
Raw Chuanwu HA	238.5(2.15%)	205.7(8.74%)
Raw Chuanwu MA	227.8(8.67%)	204.4(11.84%)
Raw Chuanwu A	41.57(18.1%)	45.5(23.8%)
Raw Caowu HA	210.3(23.02%)**	195.5(19.11%)**
Raw Caowu MA	179.4(17.18%)**	168.2(26.25%)**
Raw Caowu A	78.13(23.26%)**	49.75(27.07%)**

Key: N.D.=not detected

* n = 3

** n = 5

CHAPTER FIVE

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

On-line HPLC/APIMS is a highly selective and sensitive method for the analysis of aconitine-type alkaloids in aconite roots.

It has been shown that increasing triethylamine, ammonium acetate, or acetic acid concentration can reduce retention times of aconitine-type alkaloids. The optimized mobile phase composition would consist of 77 % buffer, 13 % acetonitrile, and 10 % tetrahydrofuran. While the buffer contains 0.5 % triethylamine, 50 mM ammonium acetate, and 1 % acetic acid in ultra-pure water. These HPLC conditions can be operated in isocratic elution such that the analysis time for a sample is less than half an hour. Then aconitine-type alkaloids can be well separated from the matrix, and simultaneously determined by UV detector.

Although ESI is a softer ionization technique than APCI, the latter would have one order of magnitude higher sensitivity than the former for aconitine-type alkaloids. In APCI mode, aconitine-type alkaloids can generate intense $[M+H]^+$ and $[M+H-CH_3COOH]^+$ ions. In addition, these alkaloids have similar fragmentation pattern under the same MS/MS conditions which can serve as a tool to distinguish aconitine-type alkaloids from other compounds.

It has been shown that DAB can be used as an internal standard for quantitative analysis of aconitine-type alkaloids in HPLC and MS simultaneously. In addition, mass spectrometer would have much lower detection limit than UV detector. However, UV detector is still useful in the quantitative analysis of the major components of aconitine-type alkaloids in aconite roots.

In conclusion, HPLC/APIMS can be used to identify and quantify aconitine-type alkaloids in aconite roots.

5.2 Future Work

In the future, the method could be evaluated more thoroughly by doing more applications. For example, in case of aconite poisoning, the sample obtained can be biological fluids such as blood or urine, or decoction which normally composed of many different herbs. Therefore, the concentration of the analytes would be quite low and the matrix is even more complex. Hopefully, on-line HPLC/APIMS may solve this problem.

ACKNOWLEDGMENT

I would like to express my sincere thanks to my supervisor, Dr. Dominic T. W. Chan, for his invaluable guidance and advice throughout the entire study. I am greatly indebted to the Director of Chinese Medicinal Material Research Centre, Chinese University of Hong Kong (CMMRC), Dr. Paul P. H. But, for providing working space, equipment, and materials.

I also wish to thank all my colleagues in CMMRC, especially Ms. A. M. Sun and Mr. K. Y. Zhou, for their helpful discussion. Thanks are extended to Ms. S. W. Cheng, Ms. Karen W. Y. Fong, Ms. Rebecca L. C. Lau, Ms. Carol K. L. Wong, Mr. Leo Y. C. Li, Mr. Eric T. P. Sze, and all my friends in the Department of Chemistry of the Chinese University of Hong Kong, for their support and encouragement during my study.

Finally, financial support from the Industrial Support Fund (AF/122/95 and AF/181/97) is gratefully acknowledged.

APPENDIX

List of the tested compounds for internal standard of aconitine-type alkaloids

Compounds	UV absorption(230 nm)	MS signal	Retention times
Alantolactone	X		
Alizarin		X	
Aloin		X	
Andrographolide		X	
Anisodamine	X		
Apigenin	√	√	X
Arctiin		X	
Arginine		X	
Atropine	√	√	X
Baicalein	√	√	X
Baicalin	√	√	X
Berberine	√	√	X
Bergenin	√	√	X
Bilirubin	√	√	X
Brucine	√	√	X
Caffeine		X	
Cantharidin		X	
Catalpol		X	
Catechin		X	

Chrysophanol		X	
Chuanxiongzine		X	
Cinobufagin		X	
Cinobufotalin		X	
Conine	√	√	X
Curcumenolum		X	
Curcumin		X	
DAB	√	√	√
Demissidine	X		
Deoxyschizandrin	√	√	X
2-(Diethylamino)-N-(2,6-dimethylphenyl)-acetamide	X		
Digitoxin		X	
1,8-Dihydroxyanthraquinone		X	
N,N-Dimethyl-5-methoxytryptamine		X	
Diosgenin		X	
DL-Alanine		X	
DL-Citruline		X	
DL-Leurine		X	
DL-Valine		X	

Dracorubin		X	
Emodin		X	
Ephedrine	√	√	X
epi-Inositol		X	
Evodiamine	√	√	X
Evodin		X	
Gastrodin		X	
Ginsenoside Re		X	
Ginsenoside Rg_1		X	
Gitoxin		X	
Gramine		X	
Honokiol	X		
Hyoscyamine	√	√	X
Icariin		X	
Imperatorin	√	√	X
Isopsoralen		X	
Jatrorrhizine	√	√	X
Jujuboside B		X	
Loganin		X	
Matrine	X		
5-Methoxypsoralen	√	√	X
4-Methoxysalicyaldehyde		X	
2-Methylnaphthalene		X	

Naringin		X	
Neoplatyphylline	√	√	X
Osthole	√	√	X
Oxymatrine	√	√	X
Paeoniflorin		X	
Paeonol	√	√	X
Palmatine	√	√	X
Panaxatriol		X	
Papaverine	√	√	X
Peimine	X		
Piperine	√	√	X
Podophyllotoxin	√	√	X
Protocatechin aldehyde		X	
PSO	√	√	√
Quercetin	√	√	X
Reserpine	X		
Rhein		X	
Rutecarpine	√	√	X
Rutin		X	
Salidroside		X	
γ-Schisandrin	√	√	X
Scopolamine	√	√	X
Sesamin		X	

Silymarin		X	
Sinomenine	√	√	X
Sipeimine	X		
Solanidine	X		
Sophoridine	√	√	X
Squalidine	√	√	X
Stachydrine	X		
Strychnine	√	√	X
Synephrine		X	
Tanshinone II A	√	√	X
Tetracaine	√	√	X
Tetrahydropalmatine	√	√	X
Tetrandrine	√	√	X

Key: √=fulfill the selection criteria of internal standard for aconitine-type alkaloids

X=not fulfill the selection criteria of internal standard for aconitine-type alkaloids

REFERENCES

1. H. F. Linskens and J. F. Jackson, *Alkaloids*, Springer Verlag, Berlin, p 2, (1994).
2. A. Mckillop, *An Introduction to the Chemistry of the Alkaloids*, Butterworth, London, pp1-18, (1969).
3. D. H. Chen, *Chung Tsao Yao* **15**, 180, (1984).
4. S. W. Pelletier and N.V. Mody, *J. Natural Products* **43**, 41, (1980).
5. F. P. Wang, *Acta Pharm. Sin.* **16**, 943, (1981).
6. Y. Chen, Y. L. Zhu and R. H. Zhu, *Acta Pharm. Sin.* **12**, 435, (1965).
7. Y. G. Wang, Y. L. Zhu and R. H. Zhu, *Acta Pharm. Sin.* **15**, 526, (1980).
8. Pharmacopoeia Committee, *Pharmacopoeia of the Peopleís Republic of China*, People's Health Publishing House and Chemical Technology Press, Beijing, Vol. 1, pp 26-28, (1990).
9. G. X. Li, *Zhong Yao Yao Li Du Li Lin Chuang*, Tianjin Technology and Translation Press, Tianjin, pp129-131, (1992).
10. H. Saito, T. Ueyama, N. Naka, J. Yagi and T. Okamoto, *Chem. Pharm. Bull.* **30**, 1844, (1982).
11. N. G. Bisset, *J. Ethnopharmacology* **4**, 247, (1981).
12. Y. P. Zhou, W. H. Liu, G. Y. Zeng, D. H. Chen, H. Y. Li and W. L. Song, *Acta Pharm. Sin.* **19**, 641, (1984).
13. Y. L. Dong, W. Z. Chen and G. S. Ding, *Acta Pharmacol. Sin.* **2**, 173, (1981).
14. Y. T. Tai, P. P. H. But, K. Young and C. P. Lau, *Lancet* **340**, 1254, (1992).
15. P. P. H. But and W. K. Kan, *Abstract Chi. Med.* **6**, 104, (1995).
16. P. Dickens, Y. T. Tai, P. P. H. But, B. Tomlinson, H. K. Ng and K. W. Yan, *Forensic Sci. Int.* **67**, 55, (1994).
17. P. P. H. But, Y. T. Tai and K. Young, *Vet. Hum. Toxicol.* **36**, 212, (1994).
18. S. C. Zhang, S. Z. Li, X. C. Gao and H. N. Yuan, *Beijing J. Trational Chi. Med.* **3**, 56, (1993).

19. H. M. Chang and P. P. H. But, *Pharmacology and Applications of Chinese Materia Medica*, Vols. 1 and 2, World Scientific Press, Singapore, pp 668-673, (1987).
20. Anonymous, *Report of the Working Party on Chinese Medicine*, Government Printer, Hong Kong, (1994).
21. K. Olafsson and K. Ingolfsdottir, *Planta Med.* **60**, 285, (1994).
22. H. Hikino, M. Murakami, C. Konno and H. Watanabe, *Planta Med.* **48**, 67, (1983).
23. K. Ito, Y. Ohyama, T. Hishinuma and M. Mizugaki, *Planta Med.* **62**, 57, (1996).
24. E. J. Zou and G. Q. Feng, *Chung Yao Tung Pao* **13**, 659, (1988).
25. B. Liu, F. Li, Z. C. Zhong, P. Chen and S. C. Zhang, *China J. Chinese Materia Medica* **19**, 220, (1994).
26. C. Ma, B. C. Cai and L. Chen, *China J. Chinese Materia Medica* **19**, 216, (1994).
27. J. Huang, X. H. Wang and T. T. Wu, *Acta Pharm. Sin.* **20**, 933, (1985).
28. Z. Z. Qian, *Chung Tsao Yao* **13**, 495, (1981).
29. W. J. Sun, S. Z. Mu and Z. M. Yanf, *Chung Yao Tung Pao* **9**, 125, (1984).
30. T. Q. Xiong, G. T. Liao and G. L. Xie, *Acta Academiae Medicine Sichuan* **16**, 24, (1985).
31. F. L. Wu, X. Hong and D. S. Su, *J. Shenyang College of Pharmacy* **5**, 19, (1988).
32. C. J. Liu, Q. Zeng, Q. Lin, D. G. Tang and J. C. Liao, *Chung Yao Tung Pao* **12**, 83, (1987).
33. K. Wang and Y. Y. Tong, *Acta Pharm. Sin.* **25**, 387, (1990).
34. H. Hikino, C. Konno, H. Watanabe and O. Ishikawa, *J. Chrom.* **211**, 123, (1981).
35. M. Z. Wang, B. L. Li and F. Y. Gao, *Acta Pharm. Sin.* **18**, 689, (1983).
36. X. H. Zhang and S. J. Wang, *Chi. J. Pharm.* **20**, 211, (1989).
37. Y. Y. Tong, *China J. Chinese Materia Medica* **15**, 106, (1990).
38. B. C. Cai, C. X. Nie, H. X. Nanbe, Z. X. Fubu and S. W. Yang, *Acta Medica Sin.* **5**, 190, (1990).

39. K. Ito, Y. Ohyama, Y. Konishi, S. Tanaka and M. Mizugaki, *Planta Med.* **63**, 75, (1997).

40. Y. Ito, T. Takeuchi and D. Ishii, *J. Chrom.* **346**, 161, (1985).

41. R. C. Willoughby and R. F. Browner, *Anal. Chem.* **56**, 26226, (1984).

42. W. M. A. Niessen and J. V. D. Greef, *Liquid Chromatography-Mass Spectrometry*, Marcel Dekker, Inc., New York, pp 59, 63-72, (1992).

43. Finnigan MAT, *TSQ/SSQ 7000 Atmosphheric Pressure Ionization System Operator's Manual*, Rev. A, (1993).

44. M. G. Ikonomou, A. T. Blades and P. Kebarle, *Anal. Chem.* **63**, 1989, (1991).

45. K. Wada, H. Bando and N. Kawahara, *J. Chrom.* **644**, 43, (1993).

46. K. Wada, H. Bando, N. Kawahara, T. Mori and M. Murayama, *Bio. Mass. Spec.* **23**, 97, (1994).

47. Finnigan MAT, *TSQ/SSQ 7000 Hardware Operator's and Service Manual*, Rev. B, (1994).

48. S. L. Zhou and M. Hamburger, *Rapid Comm. Mass. Spectrom.* **9**, 1516, (1995).

49. W. J. Loughh and I. W. Wainer, *High Performance Liquid Chromatography: Fundamental Principles and Practice*, Blackie Academic and Professinal, U. K., pp 31, 154-161, (1996).

50. W. R. Melander, J. Stoveken and C. Horvath, *J. Chrom.* **185**, 111, (1979).

54. K. Robards, P. R. Haddad and P. E. Jackson, *Principles and Practice of Modern Chromatographic Methods*, Academic Press, London, p 285, (1994).

51. A. Wehrli, J. C. Hhildenbrand, H. P. Kellerand and R. Stampfli, *J. Chrom.* **149**, 199, (1978).

52. M. Popl, J. Fahnrich and V. Tatar, *Chromatographic Analysis of Alkaloids*, Marcel Dekker, Inc., New York, p153, (1990).

53. M. E. F. Biemond, W. A. Sipman and J. Olivie, *J. Liq. Chrom.* **2**, 1407, (1979).

55. A. B. Svendsen and R. Verpoorte, *Chromatography of Alkaloids: part B Gas-Liquid Chromatography and High Performance Liquid Chromatography*, Elsevier Scientific Pub. Co., New York, pp 224-226, (1983).